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14. ABSTRACT

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration. However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to proangiogenic factors secreted by breast cancer, although at least two homeobox genes have been implicated in inducing the angiogenic phenotype in ECs. We are therefore testing the homeobox gene Gax regulates breast cancer-induced angiogenesis through its ability to regulate the expression of downstream target genes in ECs. Using an in vitro tube formation assay, we have found that Gax expression inhibits in vitro angiogenesis. Moreover, by real time quantitative reverse transcriptase PCR, we have found that Gax expression is downregulated by proangiogenic factors and, by cDNA microarray analysis, that Gax downregulates pro-angiogenic adhesion molecules in ECs and upregulates the cyclin-dependent kinase inhibitor p19INK4D. In addition, we have observed that Gax expression downregulates NF-κB-dependent gene expression in ECs and inhibits the binding of NF-κB to its consensus sequence. These observations will allow us to study the mechanism of Gax-mediated activation or repression of their expression to be studied and will form the basis for future studies that will examine in more detail the mechanism by which Gax activates downstream target genes in both ECs and breast cancer cells themselves and the detailed signaling pathways involved in this activation, specifically NF-κB, Wnt, and TGF-β signaling. Given the profound effect Gax has on endothelial cell activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Ultimately, these same techniques will be applied to other homeobox genes implicated in regulating EC phenotype during breast cancer-induced angiogenesis.

15. SUBJECT TERMS
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INTRODUCTION

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration (1-6). However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to pro- and antiangiogenic factors secreted by breast cancer cells. When we originally submitted our proposal, only two homeobox genes, *HOXD3* and *HOXB3*, had been strongly implicated in regulating tumor-induced angiogenesis (2, 7, 8). Since then, several more (*HOXD10*, *HOXB5*, *HOXA5*, *HOXA9*, *Pbx1*, and *Hex*, among others) have been added to the list of homeobox genes that influence the angiogenic phenotype in ECs (7-16). Of these three, two (*HOXD3* and *HOXD10*) have been directly implicated in regulating breast cancer-induced angiogenesis (13, 17). Because at the time we started this project, of the handful of homeobox genes implicated in regulating angiogenesis, only *Gax* seemed to show a strong restriction in its expression to cardiovascular tissues in the adult (18, 19), we originally proposed to test the hypothesis that *Gax* (18-35) also regulates breast cancer-induced angiogenesis through its ability to regulate the expression of specific downstream target genes in vascular endothelial cells (ECs). Basing our hypothesis on our preliminary data showing that *Gax* is expressed in vascular ECs and inhibits EC proliferation *in vitro*, later published as part of reference (23), we proposed to study the effect of breast cancer-secreted proangiogenic peptides and antiangiogenic therapies on *Gax* expression *in vitro* and in *in vivo* models of breast cancer angiogenesis. Next, using an adenovirus expressing *Gax* (36), we proposed to drive *Gax* expression in ECs in order to determine its effect on breast cancer-induced angiogenesis, both *in vitro* and in *in vivo* models. Finally, because few downstream targets of *Gax* had as yet been identified (29, 34, 36), we proposed to evaluate the changes in global gene expression in ECs that result from *Gax* expression in order to identify and evaluate likely downstream targets of *Gax*. Our results were to form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes, as well as the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on endothelial cell activation, we considered it likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Later, with the permission of the Army, based on evidence showing that *Gax* is expressed in mammary epithelial cells and at least some breast cancer cell lines, we altered our approach to look at *Gax* expression and activity in breast cancer and breast mammary epithelial cells themselves (see below).

BODY

Background

Like most cancers, breast malignancies are critically dependent upon their ability to induce the ingrowth of blood vessels from the host in order to grow and metastasize (37, 38). Numerous studies have found a correlation between secretion of proangiogenic molecules and increased angiogenesis with an increased likelihood of lymph node metastases and poorer prognosis in breast cancer (39, 40). Inhibition of tumor-induced angiogenesis has thus emerged in the last decade as a promising new strategy for breast cancer therapy, either alone or in combination with conventional therapies (41-44). Indeed, a recent ECOG study (E2100) it has been shown that the addition of the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab to paclitaxel improved disease free survival in patients with recurrent and metastatic breast cancer, so much so that the study was stopped and a press release made (http://www.nci.nih.gov/newscenter/pressreleases/AvastinBreast). Although the EC receptors and signaling pathways activated by proangiogenic factors secreted by breast cancer cells, such as vascular endothelial growth factor (VEGF) (45, 46) and basic fibroblast growth factor (bFGF) (45), have been extensively studied (47-49), much less is known about the molecular

biology of downstream transcription factors activated by these signaling pathways, which then activate the genes necessary for EC phenotypic changes during breast cancer-induced angiogenesis.

Homeobox genes encode transcription factors containing a common DNA-binding motif (1, 4-6, 50). Important regulators of body plan and cell fate during embryogenesis, homeobox genes also have pleiotropic roles in many cell types in the adult and can modulate cell cycle progression and arrest, cell differentiation, migration, and apoptosis (1, 3-5, 7, 51-53). As a gene family, they are thus excellent candidates to be involved in the final transcriptional control of genes responsible for the changes in EC phenotype induced by breast cancer-secreted proangiogenic factors. Until recently, little was known about how homeobox genes might influence angiogenesis. There is now evidence for their involvement in regulating the phenotypic changes ECs undergo during angiogenesis (7, 8, 13, 52, 54). For instance, one homeobox gene, *HOXD3*, induces the expression of integrin $\alpha_V\beta_3$ (55), resulting in the conversion of ECs to an angiogenic phenotype both *in vitro* and *in vivo* (7). Supporting a role for this gene in breast cancer angiogenesis are the observations that impaired *HOXD3* expression is associated with impaired angiogenesis in a mouse model (53) and increased *HOXD3* expression is observed in the vasculature of breast cancer and DCIS compared to the vasculature of the surrounding normal breast (17). Since the submission of our original proposal, two additional homeobox genes have been directly implicated in the regulation of EC phenotype during angiogenesis. In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene, *HOXD10*, inhibits EC conversion to the angiogenic phenotype (13), and has also been implicated in breast cancer angiogenesis by the observation that *HOXD10* expression is higher in quiescent vascular endothelium in the stroma than in breast cancer-associated vascular endothelium (13). Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels when embedded in Matrigel-containing sponges (13) in nude mice. Finally, other homeobox genes implicated in tumor angiogenesis include *HOXB3*, the expression of which results in an increase in capillary vascular density and angiogenesis (8); *HOXB5*, whose expression induces proliferation of angioblasts during embryonic development (56); *HOXA5*, whose expression downregulates a number of proangiogenic factors, such as VEGFR2, ephrin A1, HIF-1α and COX-2 (15); *HOXA9*, whose expression induces EphB4 and contributes to migration and tube formation (11); and, finally, *Hex*, whose expression in human umbilical vein endothelial cells (HUVECs) inhibits angiogenesis and blocks VEGF receptor signaling (54, 57).

The cardiovascular-specific homeobox gene *Gax* appears more likely to function as a negative regulator of breast cancer-induced angiogenesis in ECs, like *HOXD10*, Hex, or *HOXA5* (13, 15, 54). After isolating it from a rat aorta cDNA library (18, 58), we and others have shown that *Gax* has profound effects on cardiovascular tissues (22, 25, 26, 28, 29, 34, 36). In vascular smooth muscle cells (VSMCs) *Gax* expression is downregulated in response to mitogenic signals and upregulated in response to growth arrest signals (18, 35). Consistent with this observation, *Gax* induces G_1 cell cycle arrest (36) and can induce apoptosis in VSMCs under stress (28). Also, *Gax* overexpression inhibits VSMC migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, both of which are associated with the activated ("synthetic") state in VSMCs, as well as the angiogenic phenotype in ECs (34, 55). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (25, 26, 29, 36). Based on these observations in VSMCs, we looked for and found evidence that *Gax* mRNA is also expressed in ECs (52). This evidence led to our original concept that understanding the actions of *Gax* on downstream target genes, as well as signals that activate or repress *Gax* expression, could lead to a better understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer and thus to our hypothesis that *Gax* inhibits the phenotypic changes in ECs that occur when they are stimulated by the proangiogenic factors secreted by breast cancer cells. More importantly, we contended that the identification of downstream targets of *Gax* could identify previously unsuspected molecular targets for the

antiangiogenic therapy of breast cancer and other tumors, leading to new lines of investigation into breast cancer-induced angiogenesis and new therapies based on these observations. Thus, the studies we proposed and have undertaken with support from the Department of Defense have attempted to use *Gax* as a molecular tool to: (1) enhance our understanding of the mechanisms by breast cancer stimulates endothelial cells to become angiogenic; and (2) provide the basis for the design of antiangiogenic therapies of breast cancer targeting *Gax* or its downstream targets. In the third year of our study, our emphasis shifted to studying whether *Gax* is expressed in breast cancer cells themselves and what it does there.

Overview

Since this project began in 2003, we have made considerable progress in meeting the milestones originally proposed in our original Statement of Work. Near the end of year two, based on new data (see below) and overlap between this project and an NIH R01 that we learned that we would be awarded, we proposed changing our Statement of Work based on our observations and to eliminate overlap between the two grants, and were given permission to do so. Consequently, for this Final Report, we will list our progress based on both Statements of Work.

The reasons behind the request for a change in the Statement of Work were twofold:

1. Our laboratory made some observations that were somewhat unexpected, and therefore we wanted to alter the Statement of Work to pursue the implications of these observations during the last year of the Idea Award. These observations mostly flow from the cDNA microarray data and include (1) cDNA microarray data, now confirmed with preliminary Western blot data, indicating possible modulation of the Wnt signaling pathway by *Gax* activity; (2) cDNA microarray data, now confirmed with preliminary quantitative real time RT-PCR data implicating *Gax* in modulating the TGF-β pathway in endothelial cells; (3) the observation that *Gax* is expressed in at least one breast cancer cell line, as well as in some breast cancer specimens and breast tissue.
2. We had been notified that our R01 application to the NCI (1 R01 CA111344-01) was to be funded. The vast majority of the preliminary data used to support this R01 application had come from work entirely supported by the this award and a Career Development Award (DAMD17-02-1-0511, which recently expired), meaning that one of the stated purposes of this Idea Award (and the Career Development Award) was fulfilled. However, the R01 proposal had scientific overlap with some of the remaining tasks in the original Statement of Work, and this overlap needed to be eliminated prior to the start of funding, if possible. The reason is that, while pursuing the research tasks originally proposed for this Idea Award, we discovered an interesting connection between *Gax* and NF-κB in endothelial cells and decided to follow it. In fact, it was this mechanistic data that was most likely the major factor in our achieving a fundable score on our R01. Consequently, we proposed to alter the Statement of Work to eliminate the overlap and allow the remaining resources of DAMD17-03-1-0292 to be devoted to the

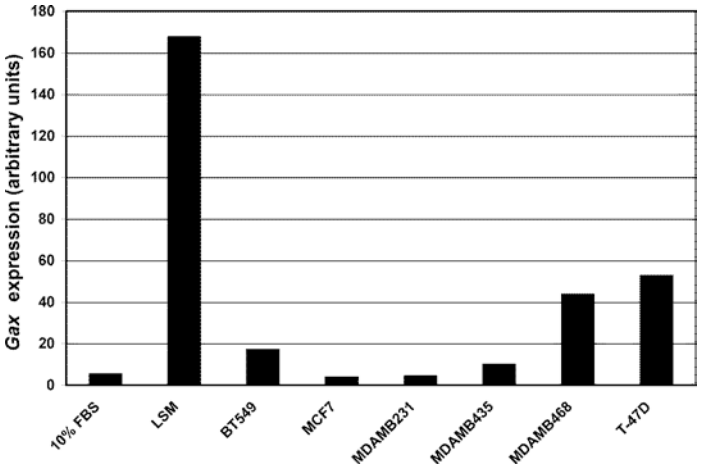

Figure 1. Downregulation of *Gax* expression in endothelial cells by conditioned medium from tumor cell lines. Quiescent HUVECs were treated with either low serum medium (LSM), 10% FBS, or 10% conditioned medium from the indicated breast cancer cell lines. Cells were harvested 4 hours after stimulation, total RNA harvested and real time quantitative RT-PCR performed. *Gax* message level was normalized to GAPDH. Units are arbitrary.

study of other promising leads regarding *Gax* regulation and function in breast cancer-induced angiogenesis and breast cancer cell proliferation not covered in the R01 application. We believe that at least two of these leads, specifically our proposal to investigate the effect of *Gax* on the Wnt and TGF-β signaling pathways in tumor vascular endothelial cells and breast cancer cells, have the potential of leading to further publications and potentially even additional applications for NIH funding.

Detailed progress report by tasks in the original Statement of Work

Task 1:Measure differences in Gax expression between angiogenic blood vessels and normal blood vessels in vivo (months 1 to 24).

a. Measure levels of proangiogenic factors in six breast cancer tumor cell lines (months 1-3)

Status: Discontinued in favor of the new Statement of Work. Because of the potentially important finding that *Gax* appears to inhibit NF-κB signaling in vascular ECs (see Task 4), we decided to defer the bulk of these experiments until Year Two. Last year, this task was eliminated in favor of other tasks in our new Statement of Work.

b. Measure breast cancer cell line-induced angiogenesis in vivo *using the Matrigel plug assay and breast cancer cell line-conditioned media, and measure* Gax *expression in endothelial cells* in vivo*. (months 1-12).*

Status: Discontinued in favor of the new Statement of Work. Although we discontinued this task in favor of tasks in the new Statement of Work, here we will provide a brief report of what has been accomplished. First, using a quantitative real time PCR assay using *Gax*-specific primers and a TaqMan probe (59), we studied *Gax* expression in ECs in response to medium conditioned by breast cancer cell lines. For nearly every breast cancer cell line we have studied, serum-free media conditioned for 24 hours by breast cancer cells strongly downregulated *Gax* expression in ECs within four hours. Two cell lines, MCF7 and MDA-MB231, were as potent as fetal bovine serum in downregulating *Gax* (Figure 1).

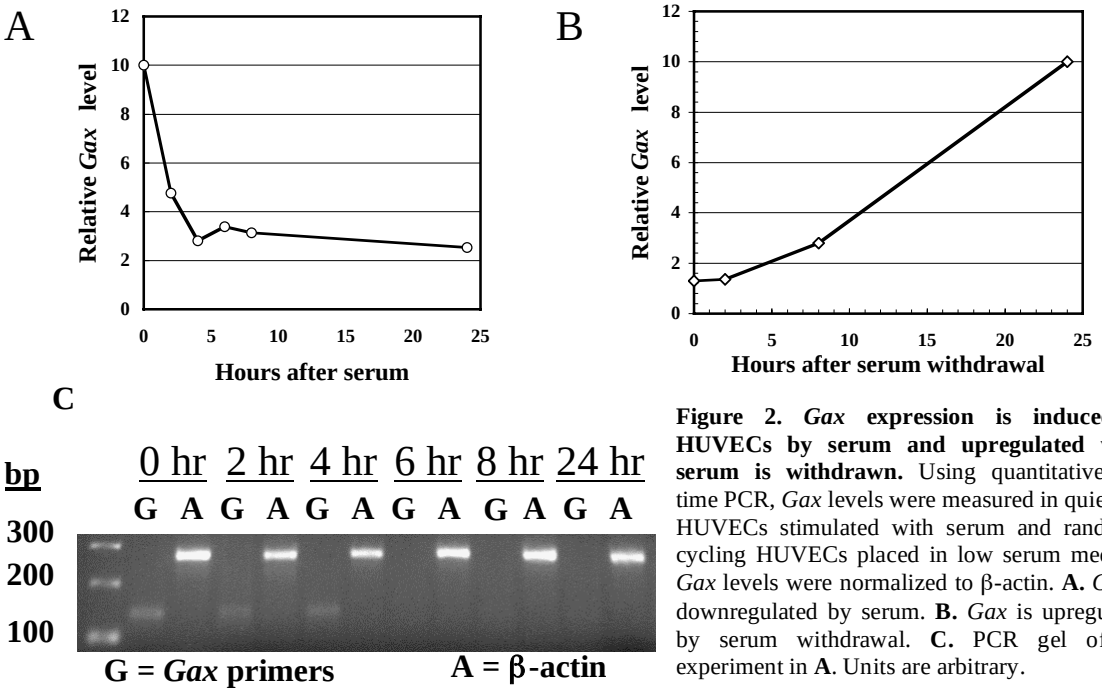

Figure 2. *Gax* expression is induced in HUVECs by serum and upregulated when serum is withdrawn. Using quantitative real time PCR, *Gax* levels were measured in quiescent HUVECs stimulated with serum and randomly cycling HUVECs placed in low serum medium. *Gax* levels were normalized to β-actin. **A.** *Gax* is downregulated by serum. **B.** *Gax* is upregulated by serum withdrawal. **C.** PCR gel of the experiment in **A**. Units are arbitrary.

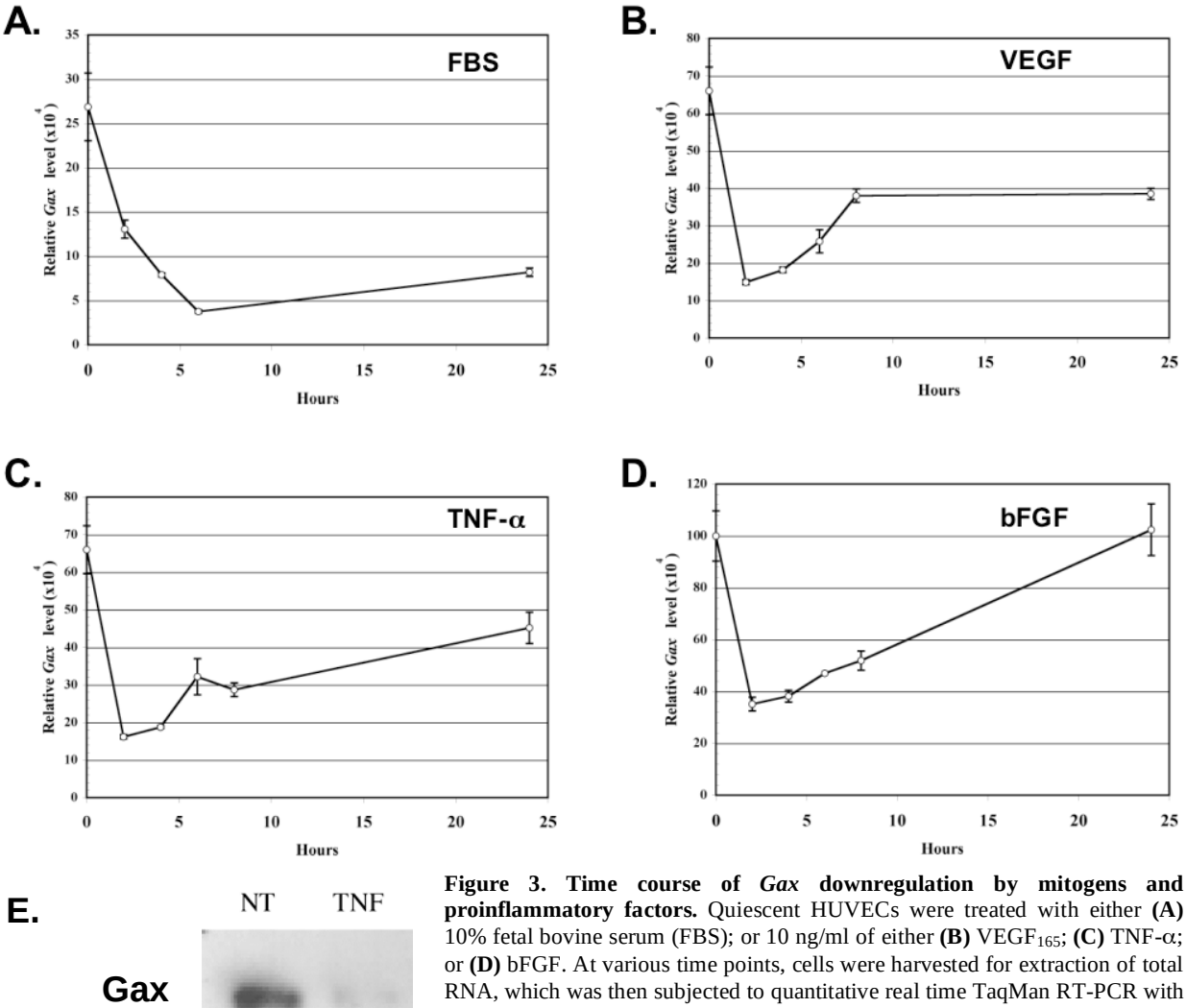

Figure 3. Time course of *Gax* downregulation by mitogens and proinflammatory factors. Quiescent HUVECs were treated with either **(A)** 10% fetal bovine serum (FBS); or 10 ng/ml of either **(B)** $VEGF_{165}$; **(C)** TNF-α; or **(D)** bFGF. At various time points, cells were harvested for extraction of total RNA, which was then subjected to quantitative real time TaqMan RT-PCR with *Gax*- and GAPDH-specific primer/probe sets. *Gax* mRNA levels were normalized to GAPDH. Units are arbitrary. E. Cells were treated identically to **(C)**, except that after six hours cells were harvested for protein extraction and then subjected to Western blot with Gax-specific polyclonal antibody.

Next, to begin identifying which factors secreted by breast cancer cells are likely to be the ones that result in downregulation of *Gax* expression, we followed up these observations by examining the effect of VEGF, bFGF, and TNF-α on *Gax* message levels using quantitative real time PCR (Figure 2). In all cases, *Gax* was rapidly downregulated and then more slowly returned to baseline after stimulation with proangiogenic factors. First, we studied the time course of *Gax* downregulation. HUVECs made quiescent by incubation for 24 hrs in 0.1% FBS were stimulated with 10% FBS plus 5 ng/ml VEGF. *Gax* was rapidly downregulated by 5-fold within four hours and slowly returned to basal over 24 to 48 hours (Figure 2, A and C). Conversely, when sparsely plated randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was upregulated nearly 10-fold within 24 hours (Figure 2B). We then stimulated quiescent HUVECs with proangiogenic or proinflammatory factors, including bFGF, VEGF, and TNF-α. *Gax* was rapidly downregulated with a similar time course (Figure 3). Similar results were observed in HMEC-1 cells, an immortalized human microvascular endothelial cell line (60) that retains many characteristics of microvascular endothelial cells (data not shown). Finally, we examined whether antiangiogenic peptides that might be used, either alone or in combination (61, 62), to treat breast cancer affected *Gax* expression. Randomly cycling HUVECs were incubated for varying

times with 1 μg/ml angiostatin (61) or endostatin (62). Both angiostatin and endostatin upregulated *Gax* expression by two-fold over 48 hours, a time course that was slower and an upregulation that was less dramatic than that caused by serum deprivation (Figure 4).

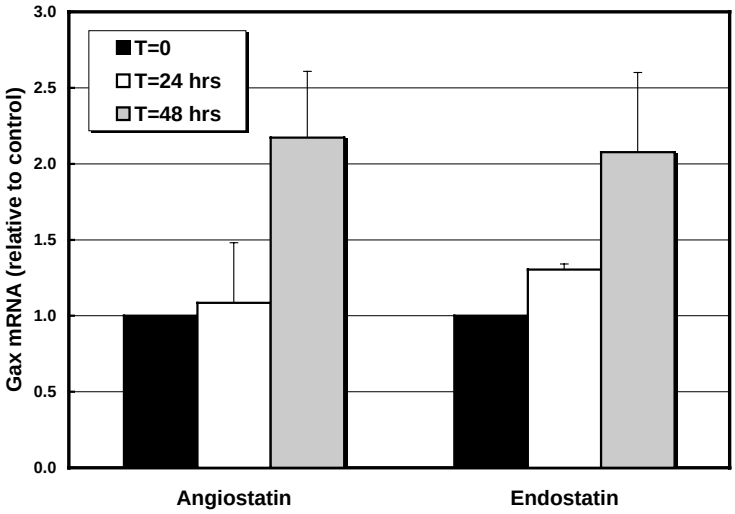

Figure 4. Upregulation of Gax by antiangiogenic peptides. Randomly cycling HUVECs were treated with either angiostatin or endostatin at 1 μg/ml. At varying time points, cells were harvested for RNA isolation, which was then subjected to reverse transcriptase quantitative real time PCR. Gax mRNA levels were normalized to GAPDH and expressed as ratios to Gax levels in control HUVECs allowed to incubate in parallel in normal medium. $p<0.01$ at 48 hrs for angiostatin and endostatin.

c. *Compare immunohistochemical staining and labeling by* in situ hybridization *for* Gax *expression in breast tumor blood vessels with that of blood vessels found in normal breast for 50 invasive human breast cancer specimens (months 12-24).*

Status: Discontinued in favor of the new Statement of Work. Although we discontinued this task in favor of tasks in the new Statement of Work, we will provide a brief report of what was accomplished. We began this task by using mouse tissues to optimize conditions for our antibody and have recently begun to do *in situ* hybridization using a probe for *Gax* that does not include its homeodomain or CAX repeat (18, 24). In order to determine if *Gax* expression *in vivo* varies according to the angiogenic state of the EC, we measured *Gax* expression *in vivo* in frozen sections of normal human breast and in human breast cancer by *in situ* hybridization. We also measured Gax protein expression in the mouse tissues from Matrigel plug experiments. In initial preliminary experiments, we observed *Gax* message expression in the capillaries and blood vessels of normal breast tissue (Figure 5, A and B). More interestingly, in a human breast cancer specimen (Figure 5C) we could also detect *Gax* expression in capillaries in the surrounding normal stroma. However, we found very few capillaries or blood vessels in the tumor itself expressing *Gax*. Consistent with this, by immunohistochemistry in frozen sections we were able to detect Gax expression in blood vessels in the skeletal muscle (Figure 5D) and stroma surrounding the Matrigel plugs (Figure 5, E and F). In contrast, the neovessels we found in the Matrigel plugs either stained weakly for Gax or not at all. We caution that these results are preliminary, but we consider them promising. Also, the frozen sections we obtained from our Tissue Retrieval Service were too thick, hence the poor tissue and cellular definition in Figure 5, A through C. These caveats aside, however, these data do at least suggest that *Gax* is regulated *in vivo* in a manner similar to how it is regulated *in vitro*, further implying a role for *Gax* in regulating *in vivo* angiogenesis. Moreover, although the thickness of the sections was too great to determine with certainty, it also appeared that the mammary epithelial cells themselves were staining positive for Gax itself on subsequent sections done after last year's report (data not shown). This work will be continued under other funding mechanisms.

Task 2: Determine the effects of Gax overexpression in endothelial cells in vitro (months 1-24).

a. *Determine effect of* Gax *overexpression and blockade on endothelial cell proliferation and expression of cell cycle regulatory genes. (months 1-12).*

Status: Discontinued in favor of the new Statement of Work. Although we discontinued this task in favor of tasks in the new Statement of Work, here we provide a brief report of what has been accomplished thus far. Using cDNA microarray experiments, we have identified several cyclin

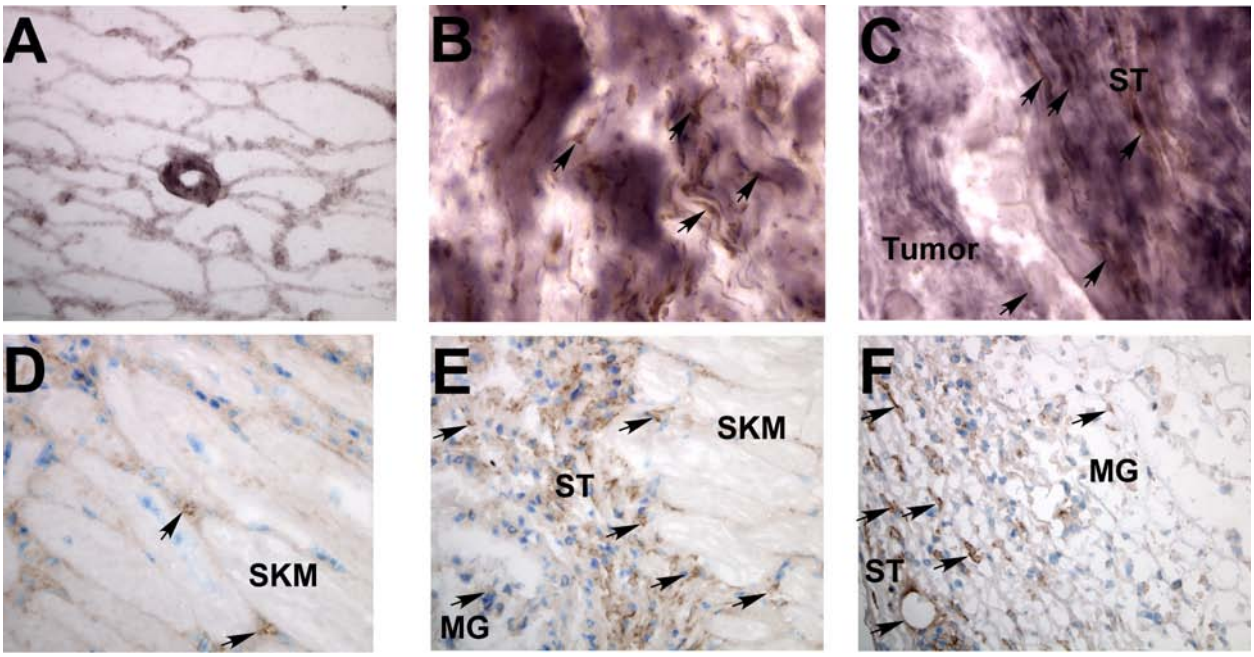

Figure 5. Determination of *Gax* expression *in vivo*. *Gax* expression was measured in human breast and breast cancer specimens by *in situ* hybridization with a riboprobe for *Gax* as described in the original grant in Specific Aim #3, p. 45 (A through C) and in Matrigel plugs harvested from mice by immunohistochemistry on frozen sections with previously described anti-Gax antibody (D through F). All photographs were taken at 400x magnification. Arrows indicate blood vessels or capillaries staining positive for *Gax* expression. (Legend: ST=stroma; SKM=skeletal muscle; MG=Matrigel plug.) **A. Normal breast (*in situ* hybridization).** In the fatty tissue of a normal human breast, a blood vessel is observed to stain positive for Gax expression. **B. Normal breast (*in situ* hybridization)**. Several capillaries stain positive for *Gax* expression. **C. Breast cancer (*in situ* hybridization).** Multiple capillaries in the stroma stain positive for *Gax* expression. However, capillaries in the tumor either stain much more weakly or do not stain positive for *Gax* at all. In addition, mammary epithelial cells in the normal breast appear to stain for Gax as well in subsequent sections (not shown). **D. Mouse skeletal muscle (immunohistochemistry).** Blood vessels in the skeletal muscle near a Matrigel plug stain positive for Gax expression. **E and F. Immunohistochemistry of control Matrigel plugs (bFGF only, no virus).** Blood vessels in the surrounding skeletal muscle or connective tissue stroma stain strongly for Gax expression, but vessels noted within the Matrigel plugs, where angiogenesis is occurring, stain either weakly or not at all.

dependent kinase inhibitors that are upregulated by *Gax* expression, including $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (36, 63, 64), and will be discussed more in the discussion of Task 4. The upregulation of these CDK inhibitors suggests redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. We have also shown that the upregulation of p21 in ECs is due to a p53-independent activity of *Gax* on the $p21^{WAF1/CIP1}$ promoter [(52), in Appendix]. Finally, we have examined the effect of *Gax* expression on the phosphorylation of ERK1/2. As can be seen in Figure 6, expression of *Gax* using our adenoviral vectors inhibits the phosphorylation of ERK1/2.

b. Determine effect of Gax *overexpression and blockade on expression of pro-angiogenic integrins, specifically if the expression of integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are regulated by* Gax *expression (Months 6-18).*

Status: Discontinued in favor of the new Statement of Work.

c. Characterize Gax*-induced endothelial cell apoptosis and the effect of* Gax *expression and*

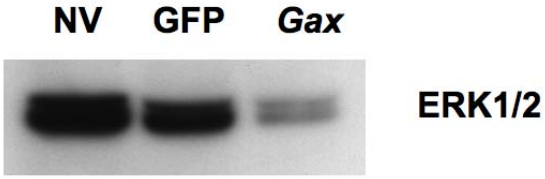

Figure 6. *Gax* blocks the phosphorylation of ERK1/2. Quiescent HMEC-1 cells pretreated with either Ad.GFP or Ad.Gax were stimulated with serum, and then cell extracts submitted to Western blot. Gax blocked phosphorylation of ERK1/2

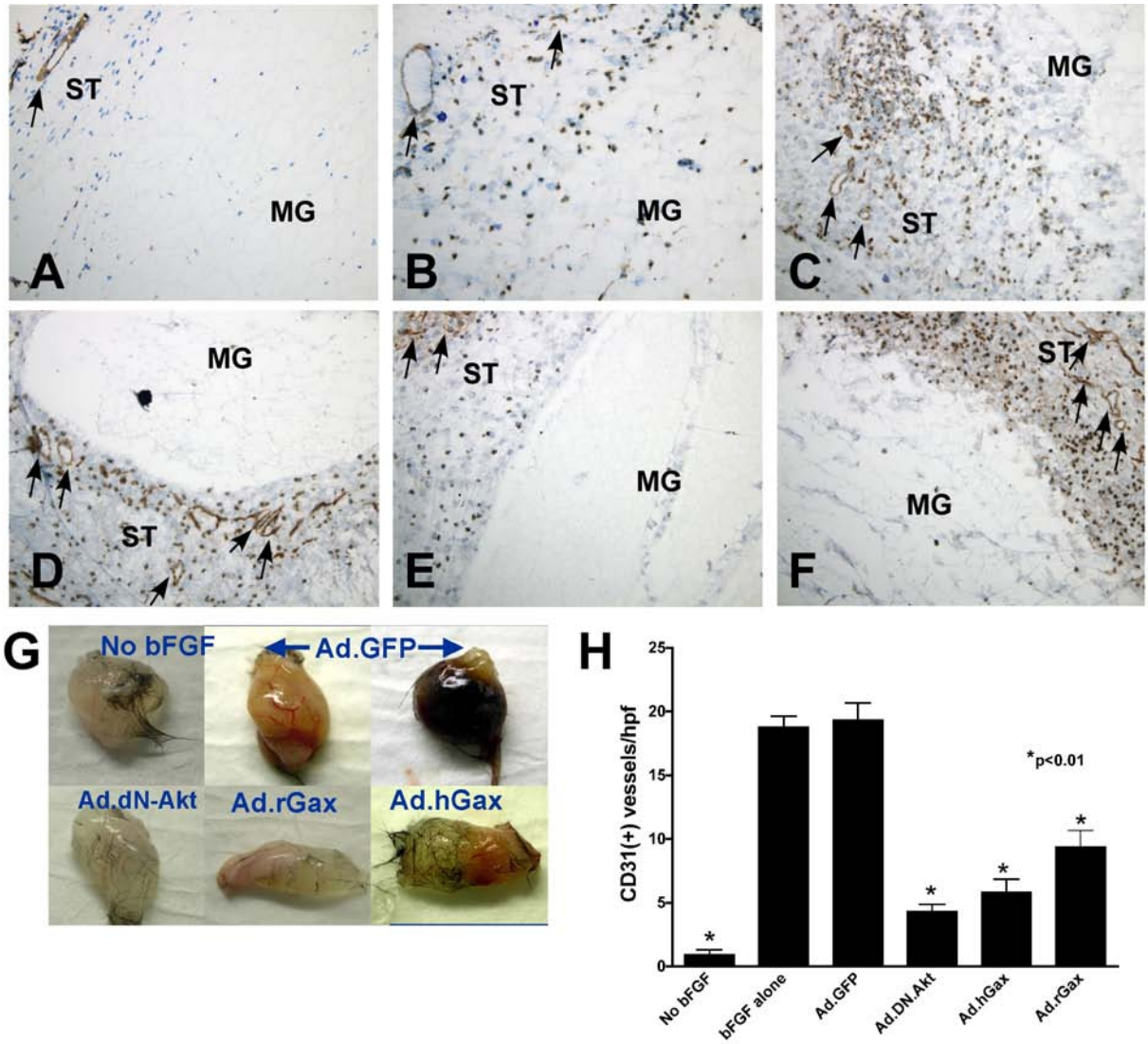

Figure 7. Effect of *Gax* expression on angiogenesis in Matrigel plugs. Matrigel plugs (500 ul each) containing 400 ng/ml bFGF and the indicated viral constructs at 10^8 pfu/plug were implanted subcutaneously in the flanks of C57BL6 mice. Plugs were harvested after 14 days incubation for immunohistochemistry using CD31 antibodies and determination of CD31-postitive cells per high powered (400x) field. Slides were photographed at 200x magnification. (**Legend:** MG = Matrigel plug; ST = stroma surrounding the plug; arrows indicate examples of CD31-positive blood vessels.) **A. No growth factor**. **B. bFGF alone, no virus. C. Ad.GFP.** Note the infiltration of the plug with CD31-positive vessels such that it is difficult to determine the exact edge of the plug in **B** and **C**. **D. Ad.dN.Akt**. **E. Ad.h*Gax***. **F. Ad.r*Gax*. G. Gross photographs of selected plugs.** Note the hemorrhage into one of the Ad.GFP plugs and the lack of vessels on the capsule of the Ad.*Gax* and Ad.dN.Akt plugs. **H. Vessel counts.** Results are plotted as means ± standard error of the mean, and statistical differences determined with one-way ANOVA $p<0.0001$ for the overall, and the vessel counts were statistically significantly different from control (Ad.GFP group) for Ad.DN.Akt ($p=0.013$); Ad.h*Gax* ($p=0.008$); and Ad.r*Gax* ($p=0.028$).

blockade on the expression of genes regulating apoptosis (months 13-24).

Status: Discontinued in favor of the new Statement of Work.

d. Determine whether Gax *expression and blockade alters the activity of two major signaling pathways implicated in endothelial cell angiogenesis (months 13-24).*

Status: Partially complete. This task has been expanded into Tasks #2 and #3 in the new Statement of Work. We have identified three potential signaling pathways that are influenced by *Gax* expression. These pathways include NF-κB (65), Wnt (66, 67), and transforming growth factor-β (68, 69). Of these, we have verified that one of them, NF-κB, is definitely inhibited by *Gax* activity, thus

completing half of this task. We will now concentrate on determining if *Gax* activity influences Wnt and transforming growth factor-β (TGF-β) signaling in ECs. See Task 4 (original S.O.W.) for a more detailed discussion of how we identified these pathways from our cDNA microarray data.

Task 3: Determine the effects of Gax overexpression on angiogenesis in vivo. (Months 13-36.)

*a. Matrigel plug assays in C57BL/6 mice to determine if Ad.*Gax *inhibits* in vivo *angiogenesis and to quantify how strong the effect is (months 13-36).*

Status: Discontinued in favor of the new Statement of Work. We discontinued this task in favor of tasks in the new Statement of Work. However, we will present a brief summary of what has been accomplished.

Matrigel containing proangiogenic factors, when implanted subcutaneously in mice, can stimulate the ingrowth of blood vessels into the Matrigel plug from the surrounding tissue, and this neovascularization can be estimated by counting CD31-positive cells and/or by measuring hemoglobin concentrations in the plug (70). Moreover, adenoviral vectors diluted in Matrigel implanted as subcutaneous plugs can serve as reservoirs to transduce ECs invading the plug and drive expression of exogenous genes (71, 72), producing effects on *in vivo* angiogenesis even when the gene transduced is a transcription factor (73). As originally proposed, we have taken advantage of this observation to test whether exogenously driven *Gax* expression can inhibit angiogenesis *in vivo*, using methodology previously described. Matrigel plugs containing bFGF and either Ad.GFP, Ad.*hGax*, or Ad.*rGax* (see manuscript in Appendix) were injected subcutaneously in C57BL/6 mice (N=8 per experimental group). As a positive control for angiogenesis inhibition by a viral vector, we utilized an adenoviral construct expressing a dominant negative form of Akt (Ad.DN-Akt) (71, 72). We observed that the adenoviral vectors expressing *Gax* expression inhibit the neovascularization of the plugs with a potency slightly less than that observed for the Ad.DN-Akt construct (Figure 7), and that the Ad.DN.Akt construct inhibited neovascularization with a potency similar to what has previously been reported (71, 72). The results of these experiments indicate that *Gax* is capable of inhibiting angiogenesis in *in vivo* models and will form the basis of experiments proposed in Task 4.

*b. Matrigel plug assays using tumor cells from breast cancer cell lines to determine if Ad.*Gax *inhibits* in vivo *angiogenesis and to quantify how strong the effect is (months 24-36).*

Status: Discontinued in favor of the new Statement of Work. We discontinued this task in favor of tasks in the new Statement of Work. The experiments encompassed by this task had not been started at the time we proposed these changes.

c. Chick chorioallantoic membrane assays to quantify Gax *inhibition of angiogenesis (months 13-36).*

Status: Discontinued in favor of the new Statement of Work. We discontinued this task in favor of tasks in the new Statement of Work. The experiments encompassed by this task had not been started at the time we proposed these changes.

Task 4: Identify potential downstream targets of Gax (months 1 through 24).

a. Construct stably transfected endothelial cells with tetracycline-inducible Gax *expression and verify inducible* Gax *expression (months 1 to 9).*

Status: Discontinued in favor of the new Statement of Work. This task has been supplanted by different tasks in the modified Statement of Work. At the time of the adoption of the new SOW, we had not yet been able to produce clones with inducible *Gax* expression.

b. Compare global gene expression between Gax-*expressing endothelial cells and non*-Gax-*expressing endothelial cells using cDNA microarrays (months 10 to 18).*

Status: Discontinued in favor of the new Statement of Work. Although we this task has been supplanted by different tasks in the modified Statement of Work, we will present briefly what has been accomplished so far. Because we were behind schedule in producing ECs with tetracycline-inducible *Gax* expression (Task 4a), we temporarily pursued a different strategy to identify changes in global gene expression due to *Gax* while we continued work on our stable transfectants. We compared global gene expression in control HUVECs infected with Ad.GFP with that of HUVECs infected with Ad.r*Gax*. Cells were infected at an MOI=100, incubated 24 hours in normal media, then harvested for total RNA isolation. Global gene expression was compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip® array set (see Methods). In general, the global changes in gene expression induced by *Gax* in this experiment were consistent with an anti-proliferative, antiangiogenic activity. There were 127 probe sets corresponding to known genes showing greater than two-fold upregulation and 115 showing greater than two-fold downregulation. Differences in gene expression between controls and *Gax*-transduced cells ranged from upregulation by approximately 30-fold to downregulation by 238-fold. This pattern was similar in ECs transduced by Ad.h*Gax*, although the magnitude of changes in gene expression tended to be smaller (data not shown). Analysis of the results was then begun (Task 4c).

c. Data analysis of cDNA microarray data to identify putative downstream targets of Gax. *(months 19-24).*

Status: Complete. We examined genes that were downregulated 24 hours after transduction of HUVECs with Ad.r*Gax* and were immediately struck by the number of CXC chemokines strongly downregulated (Table 1, which shows selected genes that are most strongly downregulated after *Gax* expression and/or most likely to be involved in angiogenesis). Most strongly downregulated of all was GRO-α (CXCL1), a CXC chemokine and a growth factor for melanoma that has also been implicated in promoting angiogenesis (74). Similarly, several other CXC chemokines were also strongly downregulated by *Gax* expression. Many of these peptides are clearly important in mediating EC activation during inflammation and in promoting angiogenesis (75). Consistent with the hypothesis that *Gax* inhibits EC activation, we also observed the downregulation of several cell adhesion molecules known to be upregulated in ECs during activation and angiogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (76, 77). These proteins have all been implicated in leukocyte-EC interactions and are upregulated by pro-inflammatory factors and by VEGF during angiogenesis (76). The pattern of downregulation of these adhesion molecules, coupled with the downregulation of CXC chemokines, suggested to us inhibition of genes normally induced by TNF-α, which in turn suggested the possibility that *Gax* may inhibit nuclear factor κB (NF-κB) activity. Indeed, when we examined our data using GeneMAPP to look for patterns of signal-dependent gene regulation (78), we found numerous NF-κB-dependent genes (65) downregulated 24 hrs after *Gax* expression (Table 1).

TABLE I: GENES REGULATED BY *GAX* EXPRESSION

UPREGULATED GENES

Genbank no.	Gene	Function	Fold change	p
L37882	Frizzled homolog 2 (FZD2)	Signal transduction	30.4	<0.0001
NM_025151	Rab coupling protein (RCP)	Signal transduction	30.1	0.0026
AI678679	Bone morphogenetic protein receptor, type IA (BMPR1A, ALK3)	Signal transduction	27.9	0.0015
N74607	Aquaporin 3 (AQP3)	Transport	19.9	0.0011
AI983115	Class I cytokine receptor	Signal transduction	12.1	<0.0001
NM_002276	Keratin 19 (KRT19)	Structural protein	9.2	<0.0001
NM_004727	Solute carrier family 24 member 1 (SLC24A1)	Ion transport	9.2	0.0007
NM_004585	Retinoic acid receptor responder (tazarotene induced) 3	Cell growth inhibition	8.5	0.0077
K01228	Proalpha 1 (I) chain of type I procollagen	Structural protein	6.4	0.0001
NM_000361	*Thrombomodulin (THBD)*	*Coagulation*	*5.5*	*0.0006*
NM_006931	Solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	Biosynthesis/metabolism	5.3	0.0000

NM_000850	Glutathione S-transferase M4 (GSTM4)	Biosynthesis/metabolism	4.9	0.0009
NM_002064	Glutaredoxin (thioltransferase) (GLRX)	Biosynthesis/metabolism	4.9	0.0001
AF162769	Thioltransferase	Biosynthesis/metabolism	4.6	<0.0001
NM_002166	*Inhibitor of DNA binding 2 (ID2)*	*Transcriptional regulation*	*4.6*	*<0.0001*
NM_017436	alpha1,4-galactosyltransferase; 4-N-acetylglucosaminyltransferase (A14GALT)	Biosynthesis/metabolism	4.3	0.0003
NM_005904	MAD (mothers against decapentaplegic) homolog 7 (MADH7)	Signal transduction	4.3	0.0006
NM_000170	Glycine dehydrogenase (GLDC)	Biosynthesis/metabolism	4.0	0.0003
NM_002222	Inositol 1,4,5-triphosphate receptor, type 1 (ITPR1)	Signal transduction	4.0	0.0000
NM_000229	Lecithin-cholesterol acyltransferase (LCAT)	Biosynthesis/metabolism	4.0	0.0002
M25915	Complement cytolysis inhibitor (CLI)	Complement activation	3.7	<0.0001
AF326591	Fenestrated-endothelial linked structure protein (FELS)	Structural protein	3.7	<0.0001
NM_001666	Rho GTPase activating protein 4 (ARHGAP4)	Signal transduction	3.7	<0.0001
NM_006456	Sialyltransferase (STHM)	Biosynthesis/metabolism	3.7	0.0001
NM_000050	Argininosuccinate synthetase (ASS)	Biosynthesis/metabolism	3.7	<0.0001
AF035620	BRCA1-associated protein 2 (BRAP2)	Biosynthesis/metabolism	3.5	0.0002
M25915	Cytolysis inhibitor (CLI)	Complement activation	3.5	<0.0001
NM_006736	Heat shock protein, neuronal DNAJ-like 1 (HSJ1)	Stress response	3.5	<0.0001
NM_000693	Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)	Biosynthesis/metabolism	3.5	<0.0001
NM_000213	*Integrin subunit, beta 4 (ITGB4)*	*Cell adhesion*	*3.5*	*0.0001*
NM_003043	Solute carrier family 6, member 6 (SLC6A6)	Transport	3.5	0.0001
AF010126	Breast cancer-specific protein 1 (BCSG1)	Unknown	3.2	0.0002
NM_005345	Heat shock 70kD protein 1A (HSPA1A)	Stress response	3.2	<0.0001
NM_006254	Protein kinase C, delta (PRKCD)	Signal transduction	3.0	0.0001
NM_000603	Nitric oxide synthase 3 (endothelial cell) (NOS3)	Biosynthesis/metabolism	3.0	<0.0001
U20498	Cyclin-dependent kinase inhibitor p19INK4D	Cell cycle	2.5	0.0004
NM_001147	*Angiopoietin 2 (ANGPT2)*	*Cell growth/chemotaxis*	*2.2*	*0.0023*
N33167	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	Cell cycle	2.1	0.0065

DOWNREGULATED GENES

NM_002167	*Inhibitor of DNA binding 3 (ID3)*	*Transcriptional regulation*	*-2.0*	*0.0081*
D13889	*Inhibitor of DNA binding 1 (ID1)*	*Transcriptional regulation*	*-2.1*	*0.0052*
NM_001546	*Inhibitor of DNA binding 4 (ID4)*	*Transcriptional regulation*	*-2.1*	*0.0056*
M60278	***Heparin-binding epidermal growth factor-like growth factor***	***Cell growth/chemotaxis***	***-2.1***	***0.0056***
NM_001955	***Endothelin 1 (EDN1)***	***Cell growth/chemotaxis***	***-2.5***	***0.0007***
NM_000201	***Intercellular adhesion molecule 1 (ICAM1)***	***Signal transduction***	***-2.5***	***0.0059***
NM_004995	*Matrix metalloproteinase 14*	*Proteolysis*	*-2.7*	*0.0002*
NM_002006	*Fibroblast growth factor 2 (basic) (FGF2)*	*Cell growth/chemotaxis*	*-2.8*	*0.0244*
NM_004428	*Ephrin-A1 (EFNA1)*	*Cell growth/chemotaxis*	*-3.0*	*0.0042*
AF021834	Tissue factor pathway inhibitor beta (TFPIbeta)	Coagulation	-3.0	0.0007
NM_016931	NADPH oxidase 4 (NOX4)	Biosynthesis/metabolism	-3.2	0.0029
NM_021106	Regulator of G-protein signalling 3 (RGS3)	Signal transduction	-3.5	0.0059
NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) (HMGCS1)	Biosynthesis/metabolism	-3.5	0.0008
NM_001146	*Angiopoietin 1 (ANGPT1)*	*Cell growth/chemotaxis*	*-3.9*	*0.0012*
NM_005658	***TNF receptor-associated factor 1***	***Signal transduction***	***-4.0***	***0.0086***
NM_001721	BMX non-receptor tyrosine kinase (BMX), mRNA	Signal transduction	-4.3	0.0007
NM_006226	Phospholipase C, epsilon (PLCE)	Signal transduction	-4.3	0.0012
NM_006823	Protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA)	Signal transduction	-4.3	0.0002
NM_002425	*Matrix metalloproteinase 10*	*Proteolysis*	*-4.4*	*0.0002*
NM_016315	CED-6 protein (CED-6)	Vesicle-mediated transport	-4.6	0.0059
NM_000600	***Interleukin 6 (interferon, beta 2) (IL6)***	***Cell growth/chemotaxis***	***-4.6***	***0.0020***
M68874	Phosphatidylcholine 2-acylhydrolase (cPLA2)	Signal transduction	-4.9	0.0007
U58111	***Vascular endothelial growth factor C (VEGF-C)***	***Cell growth/chemotaxis***	***-5.3***	***0.0020***
NM_003326	**Tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4)**	**Signal transduction**	**-5.7**	**0.0021**
AB040875	Cystine-glutamate exchanger	Biosynthesis/metabolism	-6.1	0.0012
NM_006290	***Tumor necrosis factor-α-induced protein 3 (A20, TNFAIP3)***	***Apoptosis***	***-6.4***	***0.0009***
S69738	**Monocyte chemotactic protein human (MCP-1)**	**Cell growth/chemotaxis**	**-6.5**	**0.0303**
NM_012242	Dickkopf homolog 1 (DKK1)	Signal transduction	-8.0	0.0002
NM_002852	**Pentaxin-related gene, rapidly induced by IL-1 beta (PTX3)**	**Immune response**	**-9.2**	**0.0142**
L07555	**Early activation antigen CD69**	**Signal transduction**	**-10.6**	**0.0042**
NM_001078	***Vascular cell adhesion molecule 1 (VCAM1)***	***Cell adhesion***	***-13.0***	***0.0303***
NM_002993	**Granulocyte chemotactic protein 2**	**Cell growth/chemotaxis**	**-17.5**	**0.0059**
NM_012252	Transcription factor EC	Transcriptional regulation	-18.5	0.0302
NM_000963	Prostaglandin-endoperoxide synthase 2	Biosynthesis/metabolism	-26.0	0.0303
NM_001993	***Coagulation factor III (thromboplastin, tissue factor)***	***Coagulation***	***-39.4***	***0.0022***
NM_000450	***E-selectin (SELE)***	***Cell adhesion***	***-62.6***	***0.0142***
M57731	***Chemokine (C-X-C motif) ligand 2 (CXCL2, GRO-beta)***	***Cell growth/chemotaxis***	***-79.6***	***0.0007***
NM_002090	***Chemokine (C-X-C motif) ligand 3 (CXCL3)***	***Cell growth/chemotaxis***	***-119.9***	***0.0029***
NM_000584	***Interleukin 8 (IL8)***	***Immune response***	***-181.3***	***0.0142***
NM_004591	***Chemokine (C-C motif) ligand 20 (CCL20)***	***Cell growth/chemotaxis***	***-237.6***	***0.0376***
NM_001511	***Melanoma growth stimulating activity, alpha/GRO-1/GRO-α (CXCL1)***	***Cell growth/chemotaxis***	***-238.9***	***0.0059***

Note: Boldface=genes induced by NF-κB activity; italicized=genes involved in regulating angiogenesis

The genes upregulated by *Gax* did not fall into any signal-dependent patterns as striking as the pattern of genes downregulated by *Gax* (Table 1). However, we did note results that might suggest specific pathways upregulated by *Gax*. First, there was a strong upregulation of ALK3 (bone morphogenetic receptor 1a) (79). Although it is known that, in ECs, ALK1 activates ECs through a SMAD1/5 pathway, whereas ALK5 inhibits EC activation through a SMAD2/3 pathway (68, 69), it is not known what role, if any, ALK3 plays in regulating EC phenotype. However, its upregulation by *Gax* implies that *Gax* may activate TGF-β signaling or render ECs more sensitive to TGF-β. Second, we

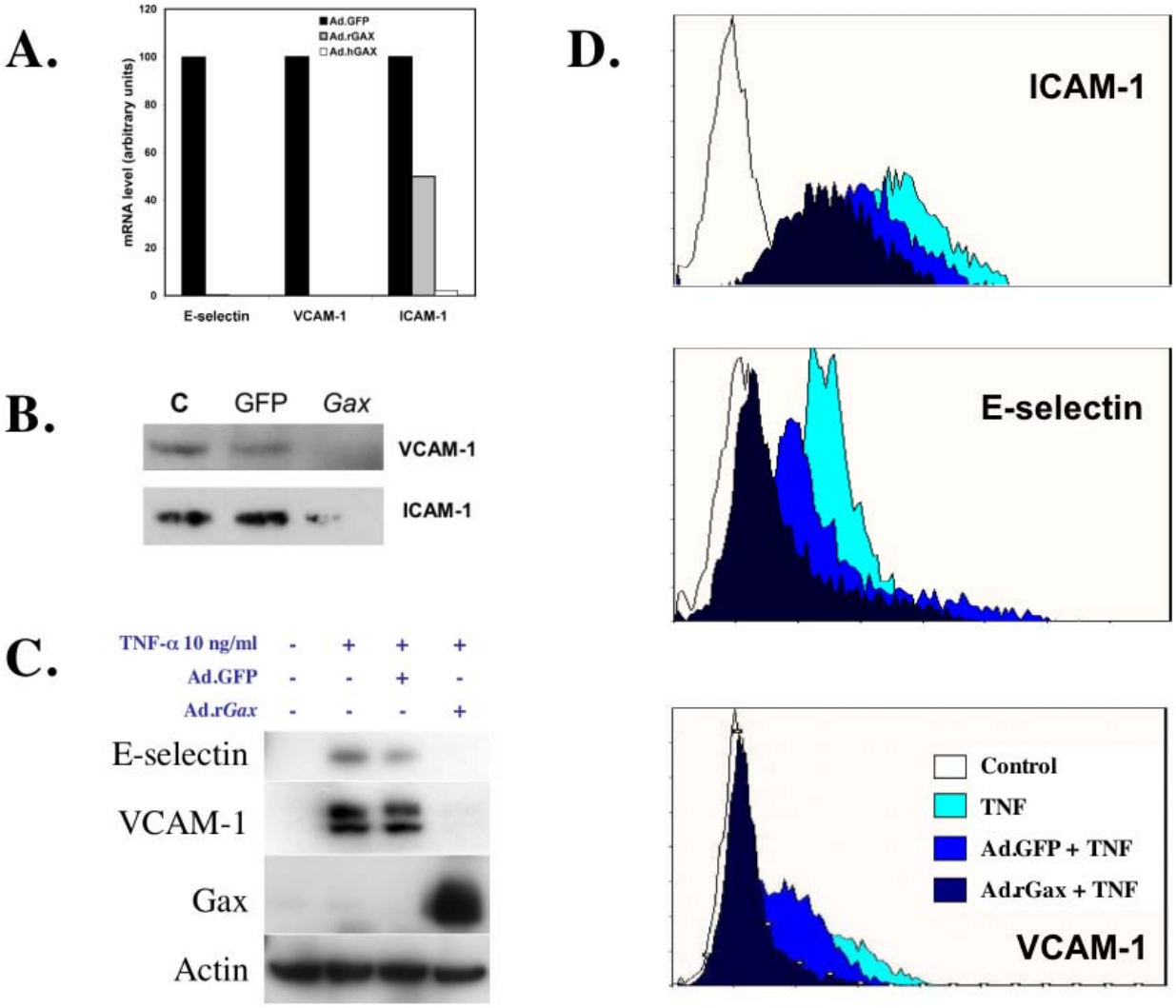

Figure 8. Effect of *Gax* expression on the level of E-selectin, VCAM-1, and ICAM-1. A. Quantitative real time PCR. Cells were harvested for total RNA isolation. Total RNA was then subjected to quantitative real time RT-PCR using TaqMan primers and probes specific for each gene and the results normalized to GAPDH. Units were chosen such that controls were set to 100. A very strong downregulation of E-selectin, VCAM-1, and ICAM-1 message level was observed. **B. *Gax* downregulates VCAM-1 and ICAM-1 proteins.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. (C= control with no virus; GFP=Ad.GFP; *Gax*=Ad.*rGax*). E-selectin could not be visualized in unstimulated HUVECs. **C. *Gax* blocks upregulation of VCAM-1 and E-selectin.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight, after which they were stimulated with 10 ng/ml TNF-α for one hour. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. Expression of Gax from the adenoviral vector was verified by Western blot with antibodies against Gax previously described. **D. *Gax* downregulates cell surface expression of ICAM-1, E-selectin, and ICAM-1**. HUVECs transduced overnight with either Ad.GFP or Ad.*rGax* at an MOI=100 were stimulated with TNF-α 10 ng/ml for 4 hours and then harvested for flow cytometry using appropriate antibodies. Ad.Gax blocked the expression of VCAM-1, E-selectin, and ICAM-1.

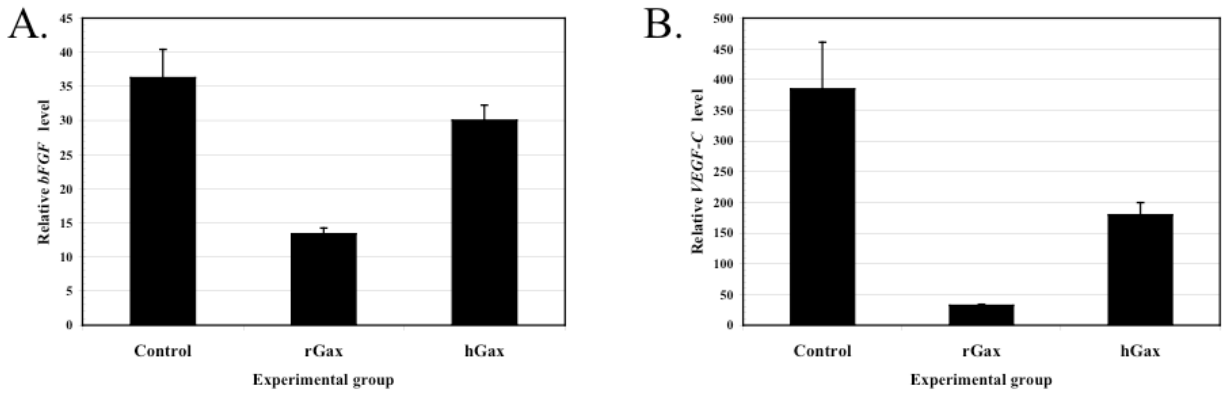

Figure 9. *Gax* downregulates proangiogenic factors expressed by ECs. HUVECs were transduced with either Ad.GFP (control), Ad.r*Gax*, or Ad.h*Gax* at MOI=100. After 24 hrs., cells were harvested for total RNA, which was then subjected to real time quantitative RT-PCR as described (Specific Aim 1). VEGF-C and bFGF message levels were normalized to GAPDH message. Units are arbitrary. **A.** bFGF. **B.** VEGF-C.

noted the upregulation of three CDK inhibitors, $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (36, 63, 64), suggesting redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. Finally, we note that *Frizzled-2* was upregulated. Little is known about the potential role of *Frizzled* receptors and Wnt signaling in regulating postnatal angiogenesis, although *Frizzled-2* is known to be expressed in ECs and there is evidence suggesting Wnt signaling inhibits EC proliferation (66, 67). This data leads us to two potential other signaling pathways besides NF-kB to pursue in Year Three.

Task 5: Verification that putative downstream targets of Gax identified by cDNA microarray are regulated by Gax (months 19 through 36).

a. Real time quantitative PCR and Western blots of genes identified in Task 4 in order to verify regulation by Gax *(months 19-36).*

Status: Discontinued in favor of the new Statement of Work. Although we this task has been supplanted by different tasks in the modified Statement of Work, we will present briefly what has been accomplished so far. Given the results of the cDNA microarray experiments, we began to pursue the task of determining whether the genes identified on the array were truly downregulated by *Gax* expression. We have now verified that a number of the genes identified in the cDNA microarray experiments as being downregulated by *Gax* are also downregulated. First, we examined several NF-κB-dependent genes, because that would represent independent verification that NF-κB signaling pathways are downregulated by *Gax* expression. We found that basal and TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin were all strongly inhibited by *Gax* expression (Figure 8). This is consistent with a role for *Gax* in inhibiting NF-κB-dependent gene expression. In addition, we noted that proangiogenic peptides such as VEGF and bFGF were also downregulated, at least at the message level (Figure 9). These observations are suggestive of a role for *Gax* in not only blocking NF-κB-dependent gene activity but for potentially blocking angiogenesis through inhibition of the autocrine stimulation of ECs.

b. Analysis of the mechanism of regulation for the most strongly regulated genes (months 19-36).

Status: Complete. Given that NF-κB activity has been implicated in the changes in phenotype and gene expression ECs undergo during angiogenesis caused by VEGF, TNF-α, and other factors, and that a number of NF-κB targets have been implicated in inducing angiogenesis (80-86), we wished to confirm the finding from cDNA microarray studies that *Gax* inhibits NF-κB activity in ECs. We therefore performed EMSAs utilizing nuclear extracts from HUVECs transduced with either Ad.r*Gax* or

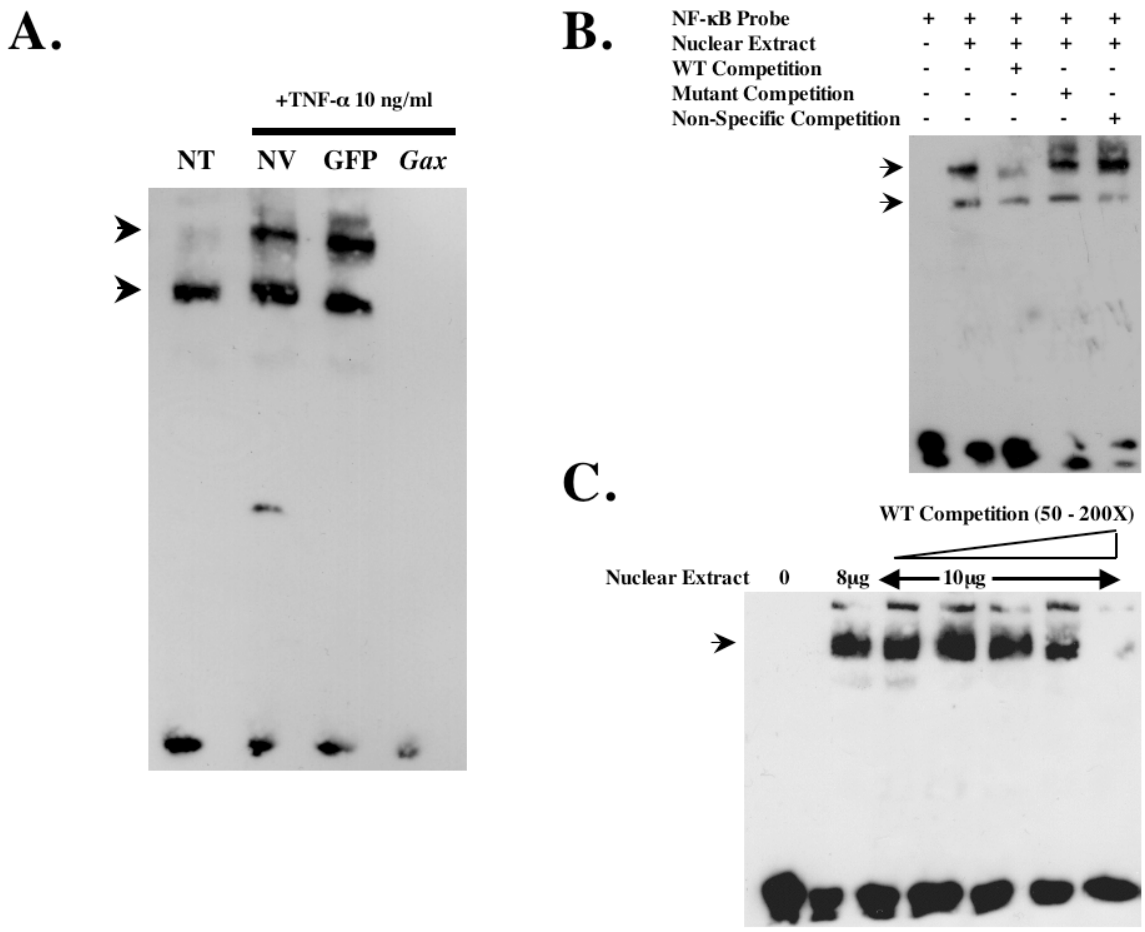

Figure 10. *Gax* expression inhibits NF-κB binding to its consensus sequence. A. Gax blocks NF-κB binding to its consensus sequence. HUVECs were infected with adenovirus containing GFP or r*Gax,* incubated overnight in EGM-2, and then induced with 10 ng/ml TNF-α for 1 hour. Controls were not induced with TNF-**α**. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce). Nuclear extracts were incubated with biotinylated oligonucleotides, containing the consensus NF-**κ**B binding site, and the reactions were electrophoresed on a 6% acrylamide gel. The reactions were transferred to positively charged nylon membrane and detected with the LightShift EMSA kit (Pierce). Arrows denote NF-**κ**B specific bands, and bands at the bottom of the gels represent unbound probe. **B and C. Control EMSAs.** These demonstrate failure of a random sequence oligonucleotide and an NF-**κ**B consensus sequence with a point mutation that abolishes DNA binding to compete with wild-type NF-**κ**B sequence **(B)** and competition with an excess of unlabeled wild-type NF-**κ**B oligonucleotide **(C)**. **Legend**: NT=no treatment; NV=no virus

the control adenoviral vector Ad.GFP to measure binding to a probe containing an NF-κB consensus sequence (87). Specific binding to NF-κB consensus sequence by nuclear extracts from HUVECs transduced with Ad.*Gax* and then induced with TNF-α (10 ng/ml) was much reduced compared to that observed in controls (Figure 10), implying that *Gax* expression interferes with the binding of NF-κB to its consensus sequence.

Next, we examined other aspects of the NF-κB signaling cascade to determine at what level *Gax* inhibits it. First, we studied the effect of *Gax* expression on an NF-κB-dependent promoter activity. Using an IL-6 promoter-Luciferase construct (88), we performed cotransfection experiments using a *Gax* expression vector (pCGN-*Gax*) and a vector expressing a truncated version of *Gax* lacking the homeodomain (pCGN-*Gax*ΔHD and measured the effect of *Gax* expression in IL-6 promoter activity. *Gax* inhibited IL-6 promoter activity in a dose-dependent fashion, an effect that was only marginally

affected by deleting the homeodomain (Figure 11). This implies that the mechanism by which *Gax* blocks NF-κB-dependent gene expression is likely not a direct competition between *Gax* and the NF-κB complex for DNA binding on the IL-6 promoter, given that the homeodomain is the DNA-binding domain of *Gax* (36). Next, we looked at the effect of *Gax* expression on IκBα degradation in response to TNF-α stimulation. HMEC-1 cells were stimulated with 10 ng/ml TNF-α, and Western blots performed at different time courses. We also found that *Gax* does not block the rapid degradation of IκBα induced by TNF-α (data not shown), implying that *Gax* is more likely to act by a direct interaction with one of the components of the NF-κB complex, rather than interacting upstream by inhibiting the degradation of IκBα or IκBβ. Although these results are very preliminary, they imply that *Gax* may actually inhibit NF-κB signaling upstream of NF-κB-dependent promoters. Indeed, over the last year, we have made several expression constructs containing *Gax* deletions, performed confocal microscopy, and coimmunprecipitation experiments that strongly suggest that the *Gax* protein interacts directly with p65 in a homeodomain-independent manner (data not shown).

Detailed progress report by tasks in the modified Statement of Work

Task 1: Identify human breast cancer cell lines that express Gax and determine if Gax regulation and function is different in them when compared to normal vascular cells (Months 25-36.)

a. *Screen a panel of 20 breast cancer cell lines for* Gax *expression by quantitative real time RT-PCR (months 25-28).*

b. *Choose the three cell lines that express the highest level of* Gax *mRNA and determine if Gax expression is downregulated by serum and mitogenic factors in the same fashion as it is in normal vascular cells (months 29-36).*

c. *Choose the three cell lines that express the lowest and highest levels of* Gax *mRNA and determine whether adenovirus-mediated* Gax *expression blocks the activity of NF-κB, as it does in vascular cells (months 29-36).*

d. *Choose the three cell lines that express the lowest and highest levels of* Gax *mRNA and determine whether adenovirus-mediated* Gax *expression inhibits cell growth, induces apoptosis (months 29-36), and/or inhibits cell invasion through Matrigel.*

Status: Partially complete. Task 1a has recently been completed. Of over 20 cell lines screened, thus far we have found four that express detectable levels of *Gax* transcript, including MCF7, ZR75-1, T47D, and BT549 cells (Figure 12 and not shown).

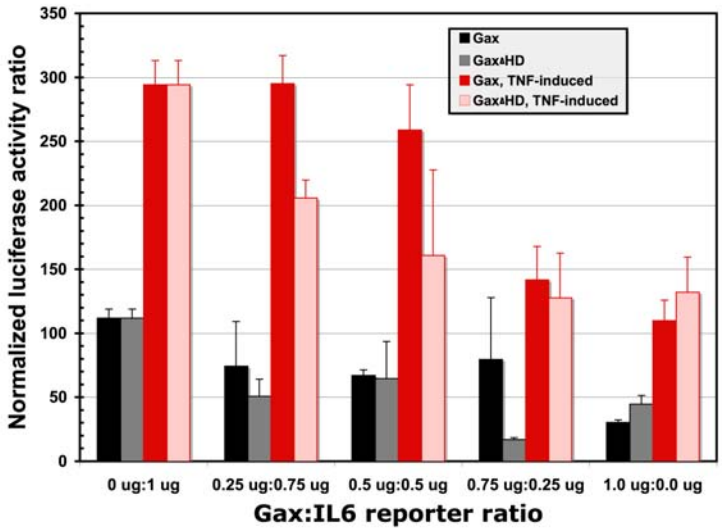

Figure 11. *Gax* expression inhibits NF-κB-dependent promoter activity. HUVECs were co-transfected with an IL-6 promoter construct plus either a vector expressing *Gax* (pCGN-*Gax*) or *Gax* lacking its homedomain (pCGN-*Gax*ΔHD) and then stimulated with TNF-α for four hours. Cells were harvested for Luciferase activity and normalized to *Renilla* Luciferase, which had been included to control for transfection efficiency. *Gax* inhibits IL-6 promoter activity, an effect that does not depend upon its homeodomain.

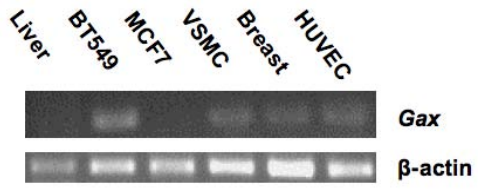

Figure 12. *Gax* is detectable by PCR in breast cancer cell lines and human tissues. RNA from tissues obtained from our Tissue Retrieval Service (breast), from mouse (liver), human ECs (HUVEC) and human breast cancer cell lines (BT549 and T47D) was subjected to PCR with Gax-specific primers. Gax is detected in two breast cancer cell lines and was also seen by Northern blot in T47D (data not shown)

This is different from previously reported assays, in which we had been unable to detect *Gax* message in MCF7 and T47D cells, but we were able to determine that our previous results were false negatives due to RNA degradation. Consequently, we are in the process of rescreening all of the previously screened cell lines in order to verify that there are no other false negatives.

For Task 1b, we next evaluated whether *Gax* is downregulated in these cells. Quiescent breast cancer cell lines (ZR75-1, BT549) were stimulated with serum and RNA harvested at different time points for QRT-PCR of *Gax* transcript levels. In all four cell lines, *Gax* expression was downregulated by serum, but there were differences from what is observed in HUVECs, HMEC-1 cells, and VSMCs. For example, in ZR75-1 cells, the downregulation was not nearly as striking as it is in normal vascular cells or even in BT549 cells or immortalized mammary epithelial cell line MCF10A (not shown). These results suggest that *Gax* may be regulated in a different manner in different breast cancer cells than it is in the vasculature, which may imply that its activity and function are different or, in the case of breast cancer, somehow lost in these cells.

Finally, we have begun experiments related to Tasks 1c and 1d. For example, we have now shown that *Gax* expression inhibits the growth of certain tumor cell lines (specifically T47D) but not others (Figure 14 and not shown). Other experiments involving measuring apoptosis, migration, and invasion, as well as activation of the NF-κB pathway in response to *Gax* are ongoing.

Task 2: Determine how Gax influences the Wnt signaling pathway in the tumor microenvironment of breast cancer, specifically in the endothelial cell compartment (as modeled in vitro with HUVECs and HMEC-1 cell), and in the tumor compartment (as modeled by the same breast cancer cell lines used in Task #1) (months 25 through 36).

a. *Quantitative real time RT-PCR of RNA and Western blots of protein extracts from* Gax*-transduced endothelial cells and tumor cells for components of the Wnt signaling pathway, including Frizzled receptors, Dsh, DKK, GSK-3α and-3β, and TCF (months 25-36).*

b. *Western blots of protein extracts from* Gax*-transduced endothelial cells and tumor cells for total and phosphorylated β-catenin*

c. *Cotransfection assays using endothelial cells and tumor cells with* Gax *expression plasmids and TopFlash and FopFlash vectors, which contain the TCF promoter coupled to Luciferase, to determine if* Gax *affects the transcription of the final downstream target of the Wnt*

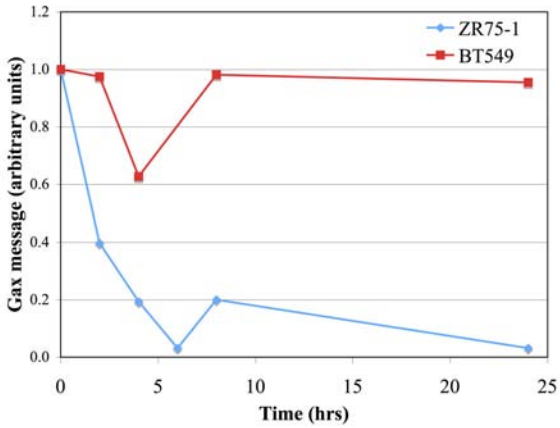

Figure 13: Gax is downregulated in breast cancer cell lines and a normal mammary epithelial cell line. Quiescent breast cancer cell lines (ZR75-1 and BT549) were stimulated with 10% FBS and then harvested at various time points for RNA for QRT-PCR to measure Gax message. Gax expression was downregulated less markedly in BT549 cells than in ECs, VSMCs, or ZR75-1 cells.

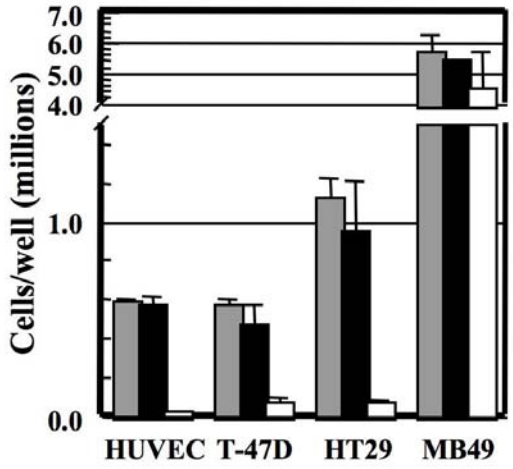

Figure 14. Effect of Gax overexpression on ECs and tumor cell lines. $3x10^5$ cells/well (HUVECs, T47D breast carcinoma, HT29 colon carcinoma, and MB49 bladder cancer cells) were plated in 6-well plates and allowed to attach. They were then infected with either empty vector (black bars) or Ad.Gax (white bars), or no virus (gray bars) at an MOI=500. Cells were incubated in growth medium for 3 days, after which they were counted. Error bars represent the standard deviation of three wells.

pathway (months 25-36).

d. Determine whether treatment of endothelial cells with Wnt ligands modulates Gax *expression (months 25-36).*

Status: Partially complete. Although Tasks 2a and 2d remain incomplete at the conclusion of this project, we have performed several experiments related to Tasks 2b and 2c thus far. First, we examined the effect of *Gax* expression on the TNF-α-induced expression of β-catenin. HMEC-1 cells were stimulated with TNF-α and then harvested for Western blot for β-catenin (Figure 15). At 45 minutes, *Gax* expression inhibited the upregulation of phosphorylated β-catenin expression compared to control, associated with a phosphorylation of JNK. Future experiments will examine the time course, ratio of phosphorylated to non-phosphorylated β-catenin, and the mechanism of this effect.

Also, we have performed several experiments thus far as part of Task 2c. Specifically, we have performed cotransfection experiments in both ECs and breast cancer cells to determine whether *Gax* can activate the β-catenin reporter using TopFlash (construct containing three TCF binding sites) and FopFlash (control containing three mutant TCF binding sites) vectors (89). First, we did these experiments in HUVECs, cotransfecting pcDNA3.1-*Gax* at various ratios to the TopFlash and FopFlash vectors. We found that *Gax* induced TCF-dependent promoter activity in ECs in a homeodomain-dependent manner (Figure 16) and that no induction was observed with the FopFlash (not shown). This result seems contradictory to the results in Figure 15 and, if verified, suggest that the regulation of Wnt signaling by Gax in ECs may be complex. We are in the process of replicating these experiments.

Task 3: Determine how Gax influences the TGF-β signaling pathway in the tumor microenvironment of breast cancer, specifically in the endothelial cell compartment (as modeled in vitro with HUVECs and HMEC-1 cell), and in the tumor compartment (as modeled by the same breast cancer cell lines used in Task #1) (months 25 through 36).

a. Western blots of protein extracts from Gax-*transduced endothelial cells and tumor cells stimulated with either BMP or TGF-β for ALK1, ALK3 (BMPR1a), and ALK5, total and phosphorylated SMAD1/5 and SMAD2*

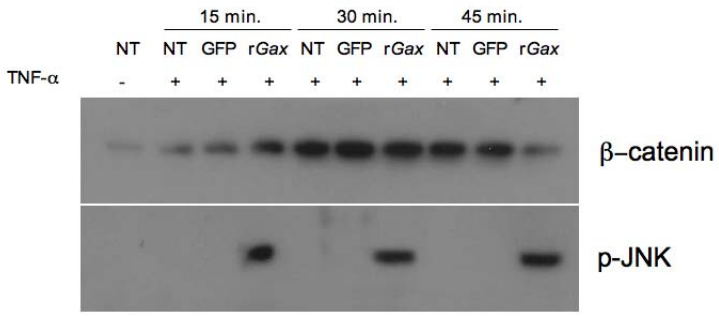

Figure 15. *Gax* inhibits β-catenin upregulation by TNF-α. HMEC-1 cells were treated with *Ad*.GFP or *Ad*.r*Gax* virus at MOI=100 for 18 hours prior to induction with TNF-α for varying times. Whole cell extracts were subjected to western blot analysis with β–catenin and a phospho specific antibody for JNK

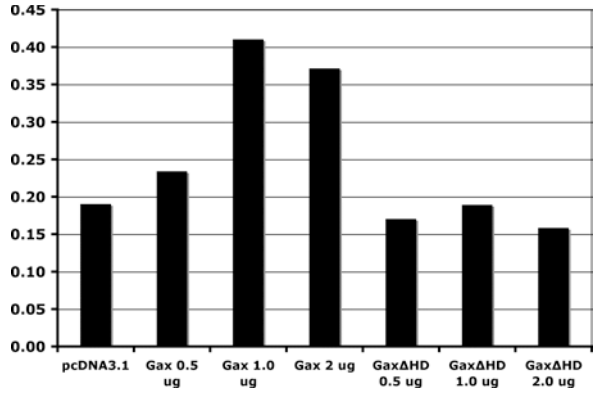

Figure 16. Gax induces β-catenin promoter activity. HUVECs were cotransfected with pcDNA3.1 vectors containing *Gax* or *Gax* lacking its homeodomain (GaxΔHD) plus the TOP-FLASH vector containing the β-catenin promoter driving Luciferase. Results were normalized to Renilla Luciferase activity (pRL-SV) to control for transfection efficiency.

to determine which pathway Gax *modulates and at what level (months 25-36).*

b. *Quantitative real time RT-PCR of the four ID gene mRNAs and Western blots of their proteins in endothelial cells transduced with Gax (months 25-36).*

c. *Determine whether TGF-β modulates* Gax *expression in vascular endothelial cells (months 25-36).*

Status: Partially complete. For Task 3a, rather than start with the Western blots, we obtained the SMAD3/4-dependent SBE-4-Luciferase promoter construct (90) and the TGF-β-induced 3TP-Luciferase promoter construct (91). Again, cotransfection experiments were carried out in HUVECs and breast cancer cell lines in order to determine whether *Gax* affected downstream targets of TGF-β. HUVECs were cotransfected with pcDNA3.1-*Gax* plus one of the two TGF-β-responsive promoters with differing ratios of *Gax* to TGF-β target, incubated for 24 hours, and then assayed for Luciferase activity using the Dual Luciferase Assay (Promega). We noted a marked activation of SBE-4-dependent Luciferase activity in HUVECs with increasing ratios of *Gax*:SBE-4 (Figure 17), implying that *Gax* is activating the SMAD3/4-dependent TGF-β pathway. Similarly, although less strikingly, we also noted increased 3TP-dependent Luciferase activity, but there appeared to be a biphasic effect, with a maximum reached, followed by a decrease in 3TP-dependent Luciferase with further increases in *Gax* construct (Figure 18). Similar, but less striking, results were observed in MCF7 and T47D cells, and further studies are ongoing to determine the differences in activation of TGF-β signaling due to *Gax* in different breast cancer cell lines. We have also completed the quantitative real time RT-PCR assays in Task 3b, confirming the results of the microarray experiment that showed that *Id1*, *Id3*, and *Id4* are downregulated by *Gax* expression (data not shown). What remains to be completed for Task 3b is the Western blotting.

Finally, Task 3c is **complete**. We studied breast cancer cell lines that expressed *Gax*, plus an immortalized mammary epithelial cell line. Cells were rendered quiescent by serum starvation and then treated with 2.5 ng/ml TGF-β, after which cells were harvested for isolation of total RNA and real time QRT-PCR to measure *Gax* transcript levels. We noted that TGF-β downregulated *Gax* expression, but in a rather interesting pattern (Figure 19). Specifically, although in some cell lines, *Gax* was strongly downregulated, while in others it was not. Moreover, in all cell lines, the downregulation of *Gax* took place over a much slower time frame, taking 8-24 hours to reach maximal

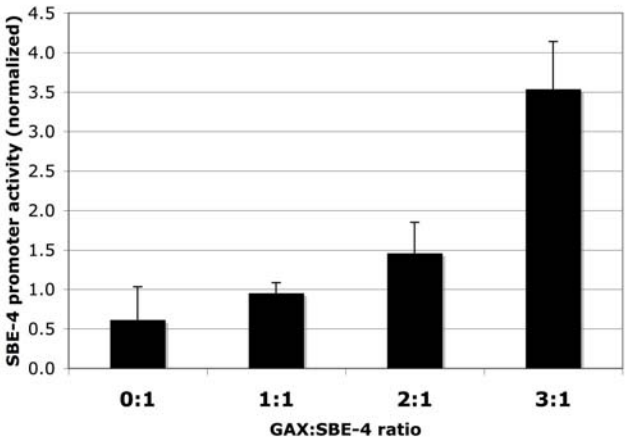

Figure 17. *Gax* activates the SBE-4 promoter. Cotransfections were carried out in HUVECs as described for the IL6 promoter construct (Figure 11) using the SMAD3/4-specific SBE-4-Luciferase vector using Lipofectin. The total DNA content of the transfection was kept constant by the addition of empty vector.

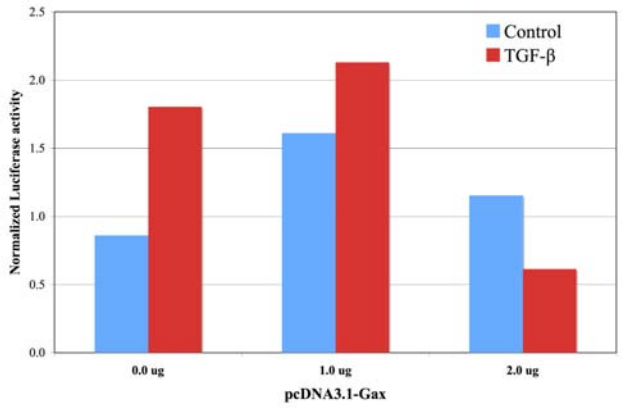

Figure 18. Gax has a weaker, biphasic effect on the activation of the 3TP promoter than on the SBE-4 promoter. Cotransfections were carried out in HUVECs as in Figure 11, except that the total DNA content of the transfection was maintained at 3 µg using empty pcDNA3.1 vector.

downregulation. These results imply that TGF-β may downregulate *Gax* by a different mechanism and that differences in the regulation of *Gax* may be involved in the pathogenesis of breast cancer. Future studies will examine this question more closely and determine whether *Gax* regulation and function are altered in breast cancer cells compared to normal mammary epithelium.

Task 4: Determine whether ERK1/2 activation or p38MAPK activation results in the downregulation of Gax (months 25 through 36).

a. Stimulate vascular endothelial cells with VEGF, angiotensin II, and bFGF and determine whether the specific ERK1/2 inhibitor PD98059 or the p38MAPK inhibitor SB203580 block the downregulation of Gax *(months 25-36).*

b. Stimulate vascular endothelial cells with VEGF, angiotensin II, and bFGF and determine whether antioxidants block the downregulation of Gax *(months 25-36).*

Status: Incomplete. We have performed experiments in Task 4b looking at whether angiotensin II and/or H_2O_2 downregulate *Gax* expression in vascular endothelial cells. Low concentrations of H_2O_2 appeared to downregulate *Gax* expression by two-fold as measured by quantitative real time RT-PCR, as did angiotensin II (not shown).

List of personnel:

	Role	**%Effort**
David H. Gorski, MD, PhD	Principle investigator	40% (no salary support)
Sejal Patel, PhD	Investigator	60% (Left the lab in July 2005
Yun Chen, Ph.D.	Investigator	60% (Replaced Dr. Patel in May 2005)
Alejandro Leal	Technician	100%

KEY RESEARCH ACCOMPLISHMENTS

Our key research accomplishments during the past three years include:

1. Demonstrated that mitogens and proangiogenic factors regulate *Gax* expression in ECs in a manner similar to that observed in vascular smooth muscle cells, with its expression maximal in quiescent cells and rapidly downregulated after ECs are treated with mitogens, VEGF, or bFGF.
2. Demonstrated that proangiogenic factors secreted by breast cancer cells downregulate *Gax* expression in ECs.

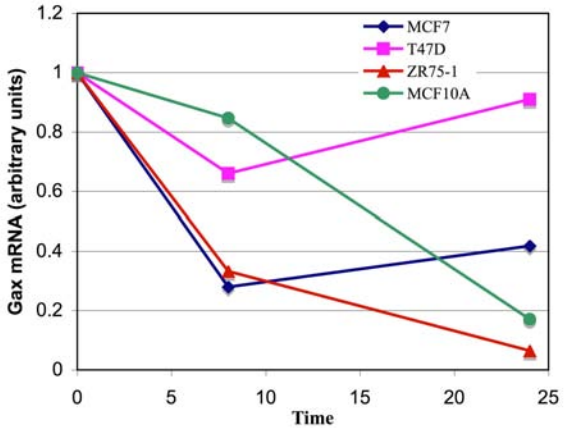

Figure 19: Gax is downregulated in breast cancer cell lines and a normal mammary epithelial cell line. Quiescent breast cancer cell lines (MCF7, T47D, and ZR75-1) and an immortalized mammary epithelial cell line (MCF10A) were stimulated with TGF-β 2.5 ng/ml and then harvested at various time points for RNA for QRT-PCR to measure Gax message. Gax expression was downregulated less markedly than in ECs or VSMCs.

3. Performed cDNA microarray experiments and began analysis of the data. This analysis shows that *Gax* downregulates the expression of NF-κB-dependent genes.
4. Confirmed cDNA microarray results for several genes identified in our initial cDNA microarray experiment at the message and protein level.
5. Demonstrated that *Gax* expression inhibits EC migration towards serum and proangiogenic stimuli.
6. Determined that *Gax* expression inhibits angiogenesis *in vivo* in the Matrigel plug assay.
7. Determined that *Gax* expression downregulates the expression of proangiogenic factors in ECs.
8. Demonstrated that antiangiogenic factors upregulate *Gax* expression in ECs.
9. Demonstrated that *Gax* expression inhibits phosphorylation of ERK1/2 in ECs.
10. Demonstrated that *Gax* expression inhibits the binding of NF-κB to its consensus binding sequence.
11. Ruled out an interaction between *Gax* and IκBα or IκBβ as a mechanism of *Gax* inhibition of NF-κB signaling.
12. Determined that *Gax* expression inhibits activation of NF-κB-dependent promoters.
13. Performed preliminary experiments demonstrating that *Gax* blocks the upregulation of β-catenin expression and may influence Wnt signaling.
14. Constructed several expression vectors to produce truncates with deletions of specific regions of the Gax protein.
15. Shown that *Gax* likely activates the SMAD3/4 pathway in both ECs and breast cancer cells.

REPORTABLE OUTCOMES

Journal articles:

1. **Gorski DH** and AD Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. *J. Surg. Res.* **111**: 91-99.
2. **Gorski DH**, and K Walsh (2003). Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc Med* **13**: 213-220.
3. Patel, S., Leal, A. D., and **D. H. Gorski** (2005). The homeobox gene *Gax* inhibits angiogenesis through inhibition of NF-κB-dependent endothelial cell gene expression. *Cancer Res.* **65**:1414-1424.
4. Chen, Y., S. Patel, A. D. Leal, and **D. H. Gorski** (2006). The homeobox gene *Gax* activates $p21^{WAF1/CIP1}$ expression in vascular endothelial cells through direct interaction with upstream AT-rich sequences. *Mol. Cell. Biol.*, submitted.

5. Chen, Y., H. Sohail, A. D. Leal, and **D. H. Gorski** (2006). The antiangiogenic homeobox gene *Gax* regulates nuclear factor-κB activity in vascular endothelial cells through a direct interaction with the p65 subunit. *In preparation.*

Abstracts

1. Patel, S., and **D. H. Gorski** (2004). Inhibition of endothelial cell activation and angiogenesis by the homeobox gene *Gax* is associated with downregulation of nuclear factor-κB (NF-κB)-dependent gene expression. *Proc. Amer. Assoc. Cancer Res.*45:77. Presented at the Annual Meeting of the American Association for Cancer Research, Orlando, FL, March 28, 2004.
2. Patel, S., and **D. H. Gorski** (2004). Inhibition of endothelial cell activation and angiogenesis by the homeobox gene *Gax* is associated with downregulation of nuclear factor-κB (NF-κB)-dependent gene expression. *Proc. Amer. Assoc. Cancer Res.***45**:77.
3. Patel, S., Y. Chen, H. Sohail, A. D. Leal, and **D. H. Gorski**. The role of the growth arrest-specific homeobox gene *Gax* in inhibiting angiogenesis and nuclear factor-κB signaling (2006). *J. Surg. Res.* **130**:162-163.

Scientific presentations at national meetings:

1. **Gorski, D. H.** *The homeobox gene* **Gax** *induces p21 expression and inhibits vascular endothelial cell activation.* The Society of Surgical Oncology Meeting, Denver, CO, March 14-17, 2002
2. Patel, S., A. Leal, and **D. H. Gorski** (2005). *Inhibition of endothelial cell activation and angiogenesis by the homeobox gene Gax is associated with downregulation of nuclear factor κB (NF-κB)-dependent gene expression.* Plenary Session, Society of Surgical Oncology Meeting, Atlanta, GA, March 3-6, 2005. Patel, S., A. Leal, and **D. H. Gorski** (2005). *Inhibition of endothelial cell activation and angiogenesis by the homeobox gene Gax is associated with downregulation of nuclear factor κB (NF-κB)-dependent gene expression.* Plenary Session, Society of Surgical Oncology Meeting, Atlanta, GA, March 3-6, 2005.
3. Patel, S., A. D. Leal, H. Sohail, and **D. H. Gorski** (2005) Inhibition of breast cancer-induced angiogenesis by a diverged homeobox gene that inhibits nuclear factor-κB-dependent gene expression in vascular endothelial cells. U. S. Army Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.
4. Patel, S., Y. Chen, H. Sohail, A. D. Leal, and **D. H. Gorski**. *The role of the growth arrest-specific homeobox gene Gax in inhibiting angiogenesis and nuclear factor-κB signaling. J. Surg. Res.* **130**:162-163. Association for Academic Surgery Plenary Session, Academic Surgical Congress, San Diego, CA, February 8, 2006.

Extramural funding applied for based on work funded by DAMD17-03-1-0292:

Source/Title	Dates	%Effort
1 R01 CA111344-01 **National Cancer Institute** **PI: David H. Gorski**	6/1/2005 - 4/30/2010	40%

Mechanism by angiogenesis inhibition by a homeobox gene

The overall goal of this project is to define more clearly the mechanism by which *Gax* inhibits endothelial cell activation and angiogenesis, specifically how it does so in vivo and how it inhibits NF-kB-dependent gene activation. A significant portion of the preliminary data used to support this grant application was obtained with the generous support of the present U. S. Army

Source/Title	Dates	%Effort
Idea Award.		
New Jersey Commission on Cancer Research **PI: David H. Gorski**	7/1/2006 - 6/30/2008	30%

Transcriptional regulation of breast cancer growth

The overall goal of this project is to study the role of *Gax* in breast cancer carcinogenesis. Status: Not funded.

CONCLUSIONS

Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult (1, 3, 4, 6, 92). It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during tumor-induced angiogenesis (7, 8, 13, 53, 54, 93). Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during breast cancer-induced angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic (94). More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3* (7). *In vivo*, sustained expression of *HOXD3* on the chick chorioallantoic membrane (CAM) retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding (53). Moreover, *HOXD3* expression is elevated in breast cancer tumor vasculature as compared to normal vasculature, as measured by *in situ* hybridization (17). More recently, overexpression of another homeobox gene, *HOXB3* has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis (8). Another example is *HOXB5*, whose expression is necessary for the expansion of flk-1-postive angioblasts during development (56). In contrast, *HOXD10* inhibits EC conversion to the angiogenic phenotype, and sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis *in vivo* (95). Consistent with this, *HOXD10* expression is decreased in breast cancer vasculature (13). Another homeobox gene, *Hex*, has a more complex role, being upregulated during angiogenesis but inhibiting EC tube formation on basement membranes (54). When combined with previous data showing high levels of *Hex* expression in proliferating vasculature had suggested that *Hex* would be more likely to induce EC proliferation and angiogenesis (93, 96), the observation that *Hex* inhibits *in vitro* angiogenesis suggests a more complex role for this gene than previously understood. Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce or inhibit the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype in breast cancer. Because blocking aberrant angiogenesis has the potential to be an effective strategy to treat or prevent multiple diseases,, understanding how downstream transcription factors integrate upstream signals from pro- and anti-angiogenic factors to alter global gene expression and produce the activated, angiogenic phenotype, will be increasingly important in developing effective antiangiogenic therapies for breast cancer.

Based on our data, we postulated that at least one additional homeobox gene, *Gax*, is also likely to have an important role in the phenotypic changes that occur in ECs during angiogenesis and therefore wanted to study its role in regulating breast cancer-induced angiogenesis. We originally isolated *Gax*

from a rat aorta library (18), and subsequently we and others found that in the adult its expression is restricted primarily to mesodermal tissues, particularly the cardiovascular system (19, 21, 52). Moreover, *Gax* expression is rapidly downregulated by growth factors and more slowly upregulated by growth arrest signals in VSMCs both *in vitro* and *in vivo* (18, 33, 35), and its expression results in cell cycle arrest (36, 52), p21 induction (36, 52), inhibition of migration (34), and modulation of integrin expression (34). *In vivo*, *Gax* expression in injured vasculature prevents the proliferative response that leads to restenosis after balloon angioplasty (25, 26, 29, 36). Based on these observations, we examined *Gax* expression in vascular ECs. We found that *Gax* is expressed in this cell type and that it has many of the same activities as in VSMCs. In addition, its expression inhibited EC tube formation on Matrigel *in vivo* (52). These observations led us to the present study, in which we wished to elucidate further the role(s) *Gax* may have in regulating angiogenesis, in particular breast cancer-induced angiogenesis. Consistent with its regulation in VSMCs, in ECs, *Gax* is rapidly downregulated by serum, proangiogenic, and pro-inflammatory factors (Figures 1, 2, and 3), and is able to inhibit EC migration *in vitro* (data not shown) and angiogenesis *in vivo* (Figure 6) These observations led us to examine the mechanism by which *Gax* inhibits EC activation utilizing cDNA microarrays to examine global changes in gene expression due to *Gax*. In addition to observing that *Gax* upregulates cyclin kinase inhibitors (Table 3) and downregulates a number of proangiogenic factors, we also found that *Gax* inhibits the expression of a number of NF-κB target genes (Table 1). Consistent with the cDNA microarray data, *Gax* inhibits the binding of NF-κB to its consensus sequence (Figure 10).

The NF-κB/Rel proteins are an important class of transcriptional regulators that play a central role in modulating the immune response and promoting inflammation and cancer by regulating the expression of genes involved in cell growth, differentiation, and apoptosis. In many cell types, NF-κB promotes cell survival in response to pro-apoptotic stimuli, induces cellular proliferation, or alters cell differentiation. Several lines of evidence have implicated NF-κB activity in regulating EC phenotype during inflammation and angiogenesis and, in particular, the classic activation of RelA-containing heterodimers (76, 80-85, 97). For example, proangiogenic factors such as VEGF (76), TNF-α (97), and platelet-activating factor (80) can all activate NF-κB signaling and activity in ECs. In addition, inhibition of NF-κB activity inhibits EC tube formation *in vitro* on Matrigel (85, 98), and pharmacologic inhibition of NF-κB activity suppresses retinal neovascularization *in vivo* in mice. (99) Moreover, ligation of EC integrin $\alpha_V\beta_3$ by osteopontin protects ECs against apoptosis induced by serum withdrawal, an effect that is due to NF-κB-dependent expression of osteoprotogerin (83). Similarly, $\alpha_5\beta_1$-mediated adhesion to fibronectin also activates NF-κB signaling and is important for angiogenesis, and inhibition of NF-κB signaling inhibits bFGF-induced angiogenesis (81). One potential mechanism by which NF-κB signaling may promote angiogenesis is through an autocrine effect, whereby activation of NF-κB induces expression of proangiogenic factors such as VEGF, as has been reported for platelet-activating factor-induced angiogenesis (80). Alternatively, the involvement of NF-κB in activating EC survival pathways is also likely to be important for sustaining angiogenesis (98).

Although NF-κB activity can influence the expression of homeobox genes (94, 100), there have been relatively few reports of functional interactions between homeodomain-containing proteins and NF-κB proteins. The first such interaction reported was between IκBα and HOXB7, where IκBα was found to bind through its ankyrin repeats to the HOXB7 protein and potentiate HOXB7-dependent gene expression (101). More recently, it was reported that IκBα can also potentiate the activity of other homeobox genes, including *Pit-1* and *Pax-8*, through the sequestration of specific histone deacetylases (102). In contrast, Oct-1 can compete with NF-κB for binding to a specific binding site in the TNF-α promoter (103). In addition, at least one interaction has been described in which a homeobox gene directly inhibits NF-κB-dependent gene expression, an interaction in which Cdx2 blocks activation of

the COX-2 promoter by binding p65/RelA (104). It remains to be elucidated if *Gax* inhibits NF-κB-dependent gene expression by a similar mechanism. Regardless of the mechanism, however, our observations made while doing the research funded by this Idea Award, to our knowledge, represent the first description of a homeobox gene that not only inhibits phenotypic changes that occur in ECs in response to proangiogenic factors, but also inhibits NF-κB-dependent gene expression in vascular ECs. These properties suggest *Gax* as a potential target for the antiangiogenic therapy of breast cancer. In addition, understanding the actions of *Gax* on downstream target genes, signals that activate or repress *Gax* expression, and how *Gax* regulates NF-κB activity in ECs is likely to lead to a better understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer. Moreover, because NF-κB has also been implicated in breast cancer carcinogenesis and aggressiveness (105, 106) and we have now detected *Gax* expression and differences in *Gax* regulation from normal in breast cancer cell lines (Figure 12), our work also suggests that *Gax* may have a role in normalizing the phenotype of mammary epithelial cells. Future work will focus on this concept.

In addition, TGF-β has been implicated in breast cancer progression, both as an inhibitor and a promoter, depending upon the specific conditions (107, 108). Moreover, there is evidence that excess production and/or activation of TGF-β by breast cancer cells can contribute to tumor progression by paracrine mechanisms involving neoangiogenesis (a process that *Gax* appears to inhibit), production of stroma and proteases, and subversion of immune surveillance mechanisms. Overall, the evidence seems to suggest that TGF-β inhibits progression in DCIS and early breast cancer but stimulates progression of metastatic breast cancer. We also note that, in addition to the evidence for their role in breast cancer progression, there is evidence for the involvement of other TGF-β receptors in regulating angiogenesis at the endothelial cell level. For instance, in ECs, ALK1 activates ECs through a SMAD1/5 pathway, whereas ALK5 inhibits EC activation through a SMAD2/3 pathway (68). The role of ALK3/BMPR1a, the gene identified on the microarray as being upregulated by *Gax*, in angiogenesis has not yet been elucidated. In addition, ID proteins, which are downstream targets of BMP/TGF-β signaling, are downregulated by *Gax* in endothelial cells. Given this background and our microarray evidence suggesting that *Gax* may influence TGF-β signaling in endothelial cells (Table I), we wished to investigate whether *Gax* truly does alter TGF-β activity in endothelial cells and whether that might contribute to its antiangiogenic effect. We have now presented evidence suggesting that Gax does affect TGF-β signaling in both the EC compartment and the tumor compartment in breast cancer (Figures 17, 18, and not shown). Future studies will determine whether the regulation of TGF-β signaling is part of the mechanism by which Gax inhibits breast cancer angiogenesis and whether its affect on TGF-β signaling influences mammary carcinogenesis or breast cancer progression and metastasis.

Finally, there is a growing body of evidence implicating the Wnt signaling pathway in breast cancer pathogenesis, as recently reviewed and reported (109-113). Other evidence linking Wnt proteins to the pathogenesis of breast cancer come from observations that the expression of different Wnt proteins is altered in breast cancer compared to normal tissue (109, 110). Wnt proteins are secreted factors that interact with the Frizzled receptors and activate signaling pathways that ultimately induce the expression of β-catenin, among other factors. It is not yet clear if Wnt signaling is pro- or anti-angiogenic, but, given that *Gax* appears to increase the level of Frizzled receptors on endothelial cells, it is not unreasonable to conclude that *Gax* influences Wnt signaling, either by increasing it or by downregulating it, resulting in a feedback loop that increases *Frizzled* receptor expression. Unfortunately, our most recent evidence is somewhat contradictory, with one line of evidence (Figure 15) suggesting that Gax inhibits Wnt signaling, while another line of evidence (Figure 16) suggests that it may activate Wnt signaling. Further experiments will be necessary to determine whether one or the other is an incorrect observation or, if both are correct, how they can be reconciled. The answer,

whatever it is, is likely to be complex, and it will be helpful to determine how Gax influences Wnt signaling in breast cancer cells. Nontheless, based on our observations thus far, we certainly consider it reasonable to examine the question of (1) whether *Gax* expression modulates Wnt signaling in tumor endothelial cells and (2) the effects of *Gax* expression in breast cancer cells themselves. These studies will form the basis of asking the question of whether *Gax*, in addition to inhibiting breast cancer-induced angiogenesis, also modulates the phenotype of breast cancer cells themselves through alterations in Wnt signaling.

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APPENDICES

Publications during period of report:

Journal articles

1. **Gorski DH** and AD Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. *J. Surg. Res.* **111**: 91-99.
2. **Gorski DH**, and K Walsh (2003). Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc Med* **13**: 213-220.
3. Patel, S., Leal, A. D., and **D. H. Gorski** (2005). The homeobox gene *Gax* inhibits angiogenesis through inhibition of NF-κB-dependent endothelial cell gene expression. *Cancer Res.* **65**:1414-1424.
4. Chen, Y., S. Patel, A. D. Leal, and **D. H. Gorski** (2006). The homeobox gene *Gax* activates $p21^{WAF1/CIP1}$ expression in vascular endothelial cells through direct interaction with upstream AT-rich sequences. *Mol. Cell. Biol.*, submitted.

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Inhibition of Endothelial Cell Activation by the Homeobox Gene *Gax*

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Background. **Angiogenesis is critical to tumor growth. *Gax*, a homeobox transcription factor whose expression in the adult is restricted mainly to the cardiovascular system, strongly inhibits growth factor-stimulated phenotypic modulation of vascular smooth muscle cells *in vitro* and *in vivo*. The function of *Gax* in vascular endothelium is unknown, but we hypothesized that it may play a similar role there. We therefore studied *Gax* expression in vascular endothelial cells and its effects on proliferation and tube formation.**

Materials and methods. ***Gax*** **expression in normal endothelial cells was examined *in vitro* by Northern blot and reverse transcriptase polymerase chain reaction and *in vivo* by immunohistochemistry. A replication-deficient adenovirus was then used to express *Gax* in human umbilical vein endothelial cells (HUVECs). HUVEC proliferation, ^{3}H-thymidine uptake, p21 expression, and tube formation on reconstituted basement membrane were measured at different viral multiplicities of infection.**

Results. ***Gax*** **mRNA was detected in HUVECs by reverse transcriptase polymerase chain reaction and Northern blot analysis and in normal vascular endothelium by immunohistochemistry. Compared with controls transduced with a virus expressing β-galactosidase, *Gax* strongly inhibited HUVEC proliferation and mitogen-stimulated ^{3}H-thymidine uptake. p21 expression in HUVECs transduced with *Gax* was increased up to 5-fold as measured by Northern blot, and p21 promoter activity was activated by 4- to 5-fold. Tube formation on Matrigel was strongly inhibited by *Gax* expression.**

Conclusions. ***Gax*** **is expressed in vascular endothelium and strongly inhibits endothelial cell activation in response to growth factors and tube formation *in vitro*. These observations suggest that *Gax* inhibits endothelial cell transition to the angiogenic phenotype in response to proangiogenic growth factors and, as a negative regulator of angiogenesis, may represent a target for the antiangiogenic therapy of cancer.**

Key Words: **angiogenesis; homeobox genes; transcription factors; vascular endothelium.**

INTRODUCTION

Vascular remodeling plays a critical role in the biology of tumors, whose growth without a blood supply is limited to less than 1 mm in diameter by diffusion of oxygen and nutrients through the interstitial fluids [1]. To overcome this limitation, tumors secrete proangiogenic factors, such as vascular endothelial growth factor (VEGF) [2] and basic fibroblast growth factor (bFGF) [3], to stimulate the ingrowth of new blood vessels [1, 4]. To form new tumor vasculature, endothelial cells undergo profound phenotypic changes, many of which are similar to the phenotypic changes tumor cells undergo when invading the surrounding stroma [1, 5, 6]. They degrade their basement membrane and invade the surrounding tissue, migrate towards the proangiogenic stimulus secreted by the tumor, and then form tubular structures and finally neovasculature [1, 7]. Although the receptors and signaling pathways activated by proangiogenic factors and cytokines have been extensively studied in endothelial cells [8, 9], much less is known about the molecular biology of the downstream transcription factors that regulate the tissue-specific gene expression controlling endothelial cell growth and differentiation and are activated by these signaling pathways. These transcription factors represent a common mechanism that can be influenced by the interaction of multiple signal-

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ing pathways and therefore might represent targets for the antiangiogenic therapy of cancer.

To understand the transcriptional control of tumor-induced angiogenesis and thereby potentially identify new ways to target it therapeutically, we decided to study the role of homeobox transcription factors in regulating the phenotypic changes that occur in endothelial cells when stimulated with proangiogenic factors. Because of their ubiquitous role as regulators of cell proliferation, migration, and differentiation, as well as body plan formation and organogenesis during embryogenesis in vertebrates and invertebrates [10, 11] and as oncogenes and tumor suppressors in various human cancers [12, 13], of all the various classes of transcription factors, we considered homeobox genes as especially likely to be important in regulating endothelial cell phenotype during angiogenesis.

Among homeobox genes, *Gax* (*G*rowth *A*rrest-specific homeobo*X*) has several characteristics that suggest it as a candidate for a role as an inhibitor of the endothelial cell phenotypic changes that occur as a result of stimulation by proangiogenic factors. Originally isolated from vascular smooth muscle [14], in the adult *Gax* expression is largely restricted to the cardiovascular system [14, 15]. In vascular smooth muscle cells, *Gax* expression is downregulated by mitogens [14, 16] and upregulated by growth arrest signals [14, 17]. Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest [18] and inhibits vascular smooth muscle cell migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_v\beta_5$ [19], both of which are associated with the synthetic state in vascular smooth muscle cells and the angiogenic phenotype in endothelial cells [19, 20]. *In vivo, Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury [21]. Because *Gax* expression is largely confined to the cardiovascular system and mesoderm-derived structures [15, 22], we considered it likely that *Gax* is also expressed in endothelial cells because endothelial cells are also derived from mesoderm. Because of its activities in vascular smooth muscle cells, we further hypothesized that *Gax* may be involved in inhibiting the phenotypic changes that occur in endothelial cells in response to stimulation with proangiogenic factors. In this report, we show that *Gax* is also expressed in vascular endothelial cells and inhibits endothelial cell cycle activation and tube formation in response to proangiogenic factors, suggesting that it has a role as a negative regulator of angiogenesis.

MATERIALS AND METHODS

Cells and Cell Culture

Human umbilical vein endothelial cells were obtained from Cambrex Biosciences (Walkersville, MD) and cultured as previously described [23] according to manufacturer's instructions in EGM-2 medium (Cambrex Biosciences, Walkersville, MD). For experiments, recombinant $VEGF_{165}$ (R & D Systems, Minneapolis, MN) was substituted in the media at the concentrations indicated for the proprietary VEGF solution.

Plasmid and Adenoviral Constructs

The *Gax* cDNA was maintained in pBluescript SK+ vectors and excised as needed for use as probes for Northern blots. Adenoviral constructs expressing the human and rat homologs of *Gax* (Ad.*hGax* and Ad.*rGax,* respectively) conjugated to the α-hemagluttinin (HA) epitope were a kind gift of Dr. Kenneth Walsh (Boston University, Boston, MA) [18], as was the control adenoviral vector expressing β-galactosidase (Ad.β-*Gal*). Both human and rat isoforms of Gax were used to verify that both isoforms have similar activity. The control adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). Viral titers were determined by plaque assay. Prior to the use of Ad.*hGax* or Ad.*rGax* in HUVECs, expression of *Gax* mRNA and protein in cells transduced with these adenoviral constructs were verified by Northern and Western blot (not shown). The p21 cDNA and p21 promoter constructs were also obtained from Dr. Kenneth Walsh and are the same constructs used in other studies [18]. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA used as a probe for Northern blots was the same construct used in another study [14].

Immunohistochemistry

Tissue sections were obtained from human surgical specimens and fixed and imbedded in paraffin according to standard procedures, with sections dehydrated through xylenes and then rehydrated through graded ethanols [15]. Staining with a polyclonal rabbit anti-Gax antibody, which labels rat, human, and mouse Gax protein, was performed according to previously described methods, except that the dilution used was 1:1000 [15]. A biotin-labeled goat anti-rabbit IgG (Sigma Corporation, St. Louis, MO) was used as a secondary antibody, and Gax staining was visualized using Vectastain ABC (Vector Laboratories, Burlingame, CA). Background staining was assessed by staining sections without primary antibody. All tissue specimens were obtained from a protocol approved by the Institutional Review Board of the University that protects the privacy of the patients from which the samples were obtained.

Northern Blots

Northern blots measuring *Gax* expression were performed as previously described [14]. Briefly, total RNA (30 μg) was isolated from cultured cells using the guanidinium thiocyanate method [24] subjected to electrophoresis through formaldehyde-containing agarose gels, capillary blotted to nylon membranes using 10× SSC as the transfer buffer, fixed to the membrane using ultraviolet crosslinking, and then hybridized to the *Gax* cDNA labeled with ^{32}P by random priming in Church buffer [25]. Blots were exposed to Kodak XAR-5 X-ray film with an intensifying screen at −80° C. Blots were then stripped with 0.1× SSC plus 0.1% SDS at 95°C and reprobed with the GAPDH cDNA to verify equal RNA loading. Hybridization temperatures were 55°C for *Gax,* p21, and GAPDH probes, and all blots were washed to a stringency of 0.2× SSC at 65°C. For p21 Northern blots, autoradiographs were scanned and band intensities determined with NIH Image v.1.6 p21 message levels were then normalized to GAPDH levels, and the fold-induction of p21 determined.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated as described above from HUVECs and used in RT-PCR to detect *Gax* transcripts. Total RNA (5 μg) was subjected to

reverse transcriptase reaction with MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexamers (Invitrogen, Carlsbad, CA). Because *Gax* has a single exon [26], all samples were treated with RNAse-free DNAse I (Ambion, Austin, TX) before being subjected to reverse transcription. As a further means of verifying that there was no genomic DNA contamination, control reactions with no reverse transcriptase were also subjected to PCR. To check the integrity of the RNA, the same reverse transcriptase reactions used to detect *Gax* were subjected to PCR using β-actin–specific primers. Human *Gax* primer sequences were: 5′-GTCAGAAGT-CAACAGCAAACCCAG-3′, sense; 5′-CACATTCACCAGTTCCTTTT-CCCGAGCC-3′, antisense; product size 247 bp, from nucleotides 566 to 812 (26). Human β-actin primer sequences were: 5′-ATCCG-CAAAGACCTGT-3′, β-actin sense; and 5′-GTCCGCCTAGAAGC-AT-3′ β-actin antisense; product size 270 bp, from nucleotides 906 to 1175 [27]. Before *Gax* primers were synthesized, their sequences were subjected to a BLAST [28] search against the Genbank database to detect any possibility that they might bind to or amplify genes other than *Gax*. Before running assays on experimental samples, each primer set, annealing conditions, Mg^{2+} concentration, and primer and probe concentration were optimized using plasmids containing the cDNA of interest. Reaction mixtures (25 μl) were used containing 0.75 U *Taq* polymerase (Gibco BRL), reaction buffer, 0.2 mM dNTPs, plus the optimized concentrations of $MgCl_2$, probe, and primers for each primer set. The PCR cycle consisted of an initial 5-min denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C (*Gax*) or 54°C (β-actin) for each primer for 60 s, and extension at 72°C for 60 s.

Cell Proliferation and ^{3}H-Thymidine Incorporation

The effect of *Gax* overexpression on mitogen-stimulated ^{3}H-thymidine incorporation was examined in HUVECs. For cell proliferation, randomly cycling HUVECs in 6-well plates (20,000 cells/plate) were transduced for 12 h with Ad.*Gax* or Ad.*β-gal* at varying MOIs, after which they were washed 3 times with phosphate-buffered saline and then placed in fresh medium EGM-2 supplemented with 10 ng/ml $VEGF_{165}$). After infection, every day 3 wells for each experimental group were trypsinized and viable cells counted, with cell viability determined by Trypan blue exclusion. For ^{3}H-thymidine uptake studies, HUVECs were made quiescent by serum starvation for 24 h in medium containing 0.1% fetal bovine serum (FBS) at which point the cells were transduced with Ad.*Gax* or Ad.*βGal* and incubated in 0.1% FBS for an additional 24 h. The cells were then stimulated with medium containing 10% FBS and 10 ng/ml $VEGF_{165}$ for 24 h in the presence of 0.2 μCi/ml ^{3}H-thymidine (Amersham, Piscataway, NJ), after which trichloroacetic acid precipitable counts were measured.

Transactivation of the p21 Promoter

Subconfluent HUVECs were plated in 6-well plates and allowed to attach for 4 h. They were then infected with different MOIs of Ad.*hGax*, Ad.*r*Gax, or Ad.*GFP* overnight, then transfected with p21 promoter Luciferase reporter construct. Transfection was performed using 2 μg p21-Luciferase plasmid per well, plus 0.2 μg pRL-SV (Promega, Madison, WI), which contains the cDNA for *Renilla reniformis* Luciferase downstream from the SV40 promoter as its reporter instead of the cDNA for firefly Luciferase, as a control for transfection efficiency. Firefly and *Renilla* Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI), and the firefly Luciferase activity from the p21-Luciferase promoter construct normalized to the constitutive *Renilla* Luciferase activity from the pRL-SV plasmid.

Tube Formation Assay

Tube formation assays were performed essentially as described [29]. Briefly, HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*), or GFP (Ad.*GFP*) at various multiplicity of infection (MOI). Eighteen hours later 5×10^5 cells were plated on 6 well plates whose surfaces had been coated with reconstituted basement membrane, Low Growth Factor Matrigel, (BD Biosciences, San Jose, CA) and incubated overnight in the presence of serum and 10 ng/ml $VEGF_{165}$. After this, the number of tubes per high-powered field were counted for 10 high-powered fields, with tubes being defined as a completed connection between cells. Ad.GFP-transduced cells were also examined using a fluorescence microscope to demonstrate that GFP was being expressed in the HUVECs forming tubes.

Data Analysis and Statistics

Experiments were repeated 3 or more times. For cell culture experiments, at least three wells per experimental group were measured and the mean ± standard deviation determined. Statistical significance between the various groups was determined by 2-way ANOVA and the appropriate post-test, with the results being considered statistically significant when $P < 0.05$.

RESULTS

Gax is Expressed in Human Vascular Endothelium

Because we hypothesized that Gax is expressed in endothelial cells as well as vascular smooth muscle cells, we first examined *Gax* expression in cultured human vascular endothelial cells and detected *Gax* expression in HUVECs by Northern blot (Fig. 1A) and by RT-PCR using human *Gax*-specific primers (Fig. 1B). Next, to verify that Gax protein is expressed in the endothelium of normal human blood vessels, we subjected a section of human kidney from a nephrectomy specimen to immunohistochemistry with a polyclonal rabbit anti-Gax antibody [15] (Fig. 2). As expected, *Gax* was expressed in vascular smooth muscle cells. In addition, it was also expressed in the endothelial cells lining the lumen of arteries, as evidenced by nuclear staining of the cells of the intima. From these observations, we conclude that *Gax* is expressed in normal endothelial cells, both *in vitro* and *in vivo*.

Gax Inhibits HUVEC Proliferation *in Vitro*

To test the hypothesis that *Gax* expression inhibits proliferation of endothelial cells, we transduced HUVECs that had been sparsely plated on plastic in 6-well plates with Ad.*hGax* at increasing MOI. Viable cells were counted from each experimental group every 24 h for 4 days. Control cells were transduced with Ad.*β-gal*. Up to MOI = 1000, Ad.*β-gal* did not inhibit HUVEC proliferation (data not shown). Both Ad.*hGax* and Ad.*rGax*, however, inhibited HUVEC and proliferation in a dose-dependent fashion compared to Ad.*β-gal* (Fig. 3A and B; $P < 0.05$ for all MOI of virus). Quiescent HUVECs were then transduced with either

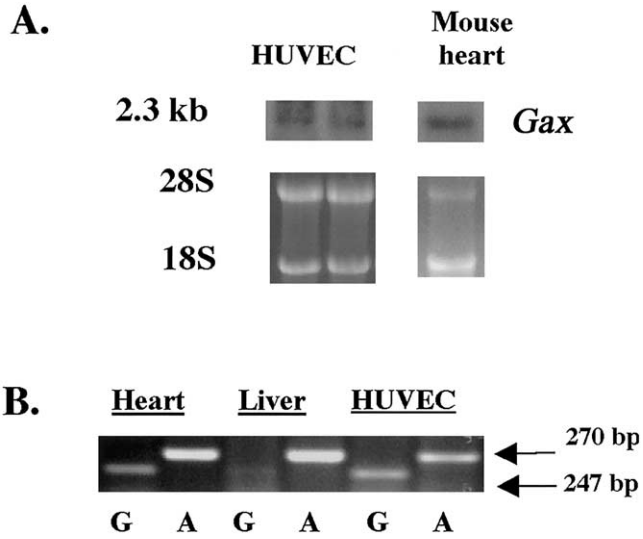

FIG. 1. Gax expression in vascular endothelial cells. Total RNA from HUVECs was subjected to Northern blot with the *Gax* cDNA labeled with ^{32}P by random priming. (A) Northern blots. Two different HUVEC preparations were studied and compared to mouse heart (MH), which is known to express *Gax*. (B) RT-PCR. Total RNA from HUVECs was subjected to RT-PCR using primers that amplify a 247-bp fragment (base 566 to 812) of the human *Gax* cDNA. The same RT reactions were also subjected to PCR using β-actin primers. See Materials and Methods for details. (G = *Gax*; A = β-actin).

Ad.*hGax* or Ad.*β-gal,* maintained in low serum medium for 24 h, then stimulated with 10% FBS and $VEGF_{165}$ = 10 ng/ml, and 24-h ^{3}H-thymidine uptakes measured (Fig. 4). For comparison, one experimental group was left in low serum medium and is labeled "Quiescent." Consistent with its effect on randomly cycling HUVECs. *Gax* strongly inhibited mitogen-stimulated ^{3}H-thymidine uptake ($P < 0.05$ for all MOI of virus). From these results, we conclude that *Gax* expression results in inhibition of HUVEC proliferation, as well as cell cycle arrest.

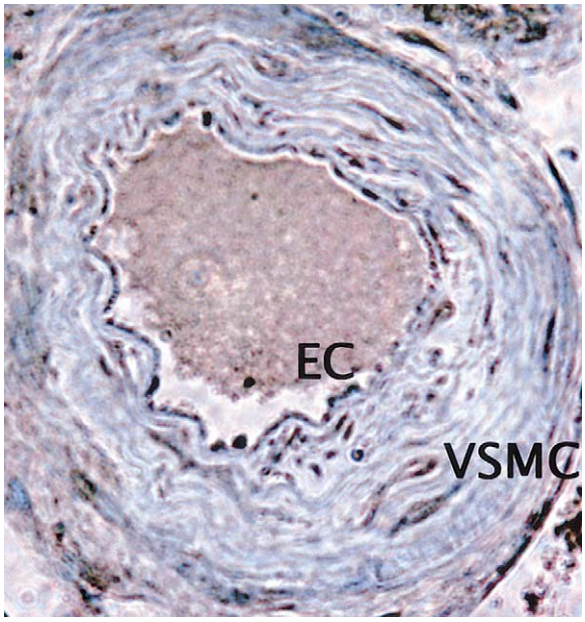

FIG. 2. *Gax* is expressed in both the vascular smooth muscle cells and the endothelial cells of normal human arteries. A section from human kidney obtained from a nephrectomy specimen for renal cell carcinoma was stained with rabbit polyclonal anti-Gax antibody. In the section containing normal kidney, Gax expression was noted in both the media, containing vascular smooth muscle cells (VSMC), as expected from previous studies, but there was also strong staining in the endothelial cells (EC) in the intima lining the lumen.

Gax Activates p21 Promoter Activity in Endothelial Cells

Because *Gax* induces p21 in vascular smooth muscle cells and *Gax* expression inhibited HUVEC proliferation as measured both by cell counts and ^{3}H-thymidine uptake, we tested whether *Gax* could induce p21 expression in endothelial cells. HUVECs were transduced with Ad.*hGax* and Ad.*rGax* at varying MOIs. Cells transduced with an adenovirus expressing green fluorescent protein (Ad.*GFP*) served as controls. By Northern blot, p21 levels were strongly induced in a viral MOI-dependent fashion (Fig. 5A). When cells transduced with Ad.*hGax* in a similar fashion were transfected with a plasmid containing the p21 promoter fused upstream to the firefly Luciferase gene, it was similarly observed that p21 promoter activity was increased by up to 7-fold (Fig. 5B; $P < 0.05$ for all MOI). Transduction with Ad.*GFP* did not affect p21 promoter activity (Fig. 5A and B), nor did transduction with Ad.*β-Gal* (data not shown).

Gax Inhibits Endothelial Cell Tube Formation on Reconstituted Basement Membranes

We next studied the effect of *Gax* expression on angiogenesis *in vitro.* HUVECs were transduced with

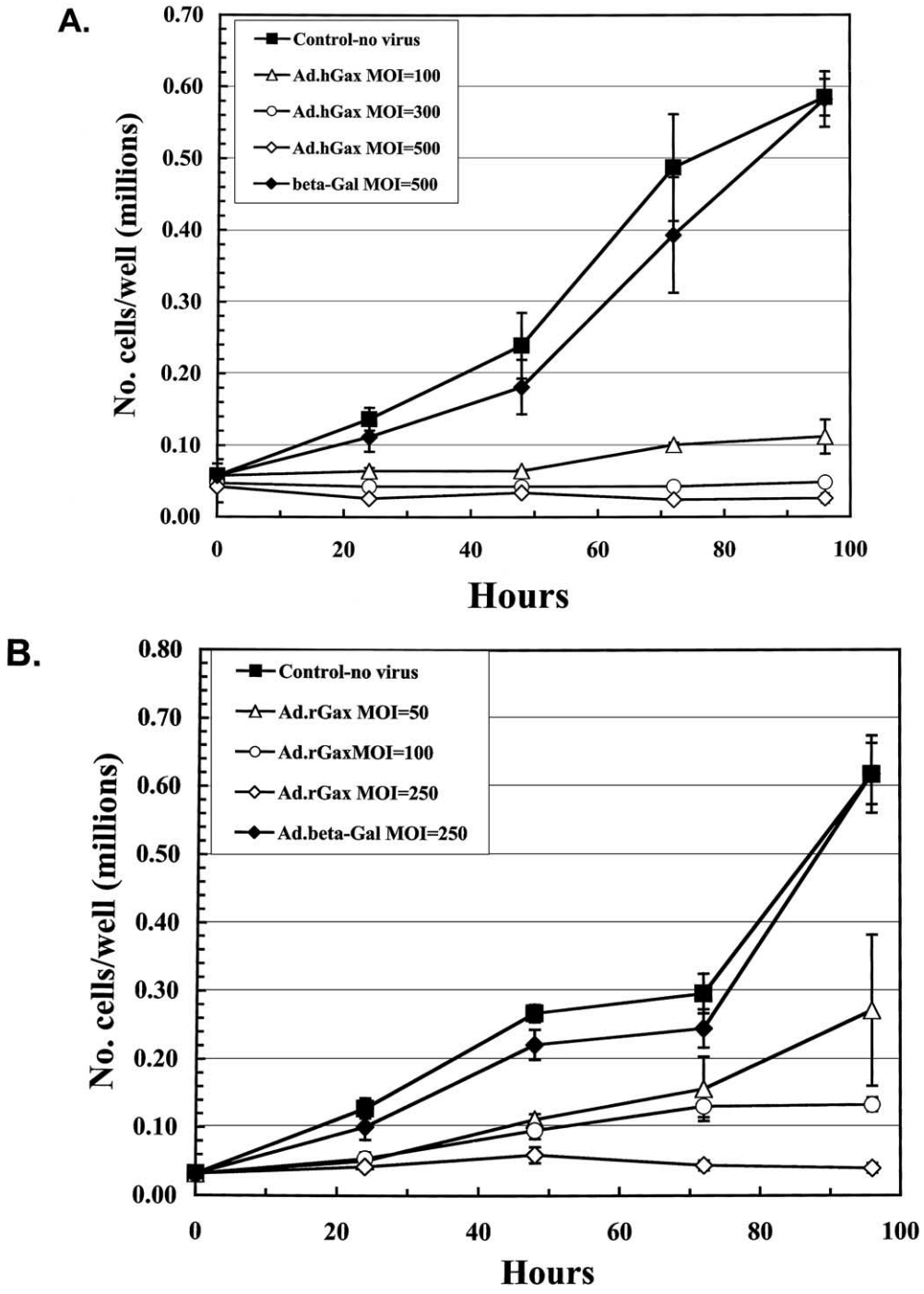

FIG. 3. Inhibition of HUVEC proliferation by *Gax.* Randomly cycling HUVECs growing in 6-well plates in EGM-2 medium were infected with varying MOI of either Ad.*hGax,* Ad.*rGax,* or Ad.*β-Gal.* After infection, 3 wells for each experimental group were trypsinized and counted, with cell viability determined by Trypan blue exclusion, and results were counted as mean number of cells ± standard deviation. Inhibition of proliferation was statistically significant for all experimental groups at all time points from 48 hours on ($P < 0.05$). (A) Effect of Ad.*hGax* on HUVEC proliferation (B) Effect of Ad.*rGax* on HUVEC proliferation.

Ad.*hGax* and Ad.*rGax* at varying MOIs and plated on reconstituted basement membrane (Matrigel) in the presence of serum and 10 ng/ml $VEGF_{165}$, conditions that result in robust tube formation. Ad.*GFP* had no effect on tube formation up to MOI = 250, and expression of GFP was verified by fluorescence microscopy (Fig. 6). However, there was a dose-dependent decrease in tube formation beginning at relatively small doses of virus (MOI = 25) and becoming maximal at MOI = 100 (Fig. 6). Maximal inhibition oc-

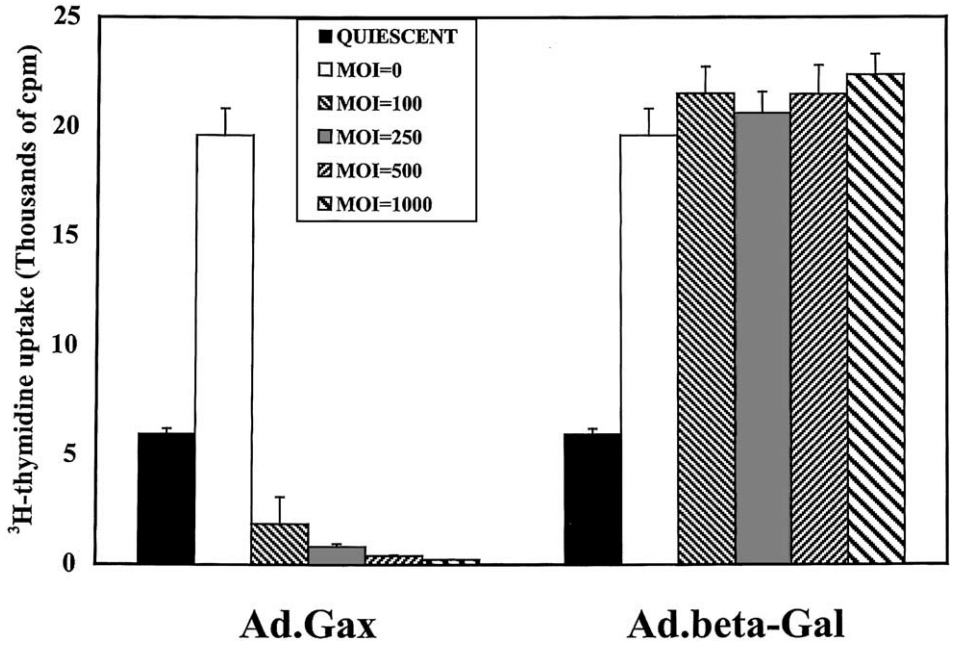

FIG. 4. Inhibition of mitogen-induced ^{3}H-thymidine uptake in HUVECs by *Gax.* Quiescent HUVECs were transduced with Ad.*hGax* at various MOI. Twenty-four hours later, the cells were stimulated with serum and $VEGF_{165}$ (10 ng/ml) and 24 h. ^{3}H-thymidine uptakes measured after stimulation. *Gax* strongly inhibited ^{3}H-thymidine uptake in response to mitogen stimulation.

curred at a lower MOI than is necessary to maximally inhibit endothelial cell proliferation and activate p21 expression and became maximal at MOI = 50 to 100. We note that is the dose range of virus that we have determined to be necessary to transduce 100% of HUVECs (not shown), implying that few viral particles per cell are necessary to produce sufficient Gax protein to inhibit the cellular machinery that causes tube formation. This is in contrast to the higher viral MOI necessary to produce maximal inhibition of cell cycle progression and induction of p21 expression, implying that more viral particles per cell and therefore a higher level of Gax protein are required to mediate these effects.

DISCUSSION

The primary target of proangiogenic factors secreted by tumor cells, and many antiangiogenic factors, is the vascular endothelial cell [1, 30]. During angiogenesis, whether physiologic or tumor-induced, endothelial cells undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix [1, 4, 31]. Endothelial proliferation accompanies cell invasion and migration, and lumens of new capillaries are formed when endothelial cells adhere to one another and form tubes. Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult [10–13]. It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during angiogenesis.

Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al.* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic [32]. More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3.* Stimulation of endothelial cells with bFGF induces *HOXD3* expression, as well as integrin $\alpha_v\beta_3$ and the urokinase plasminogen activator, effects that are blocked by *HOXD3* antisense. *In vivo,* sustained expression of *HOXD3* on the chick chorioallantoic membrane retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas [33]. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding [34]. More recently, overexpression of another homeobox gene, HOXB3, in the chick chorioallantoic has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis [35].

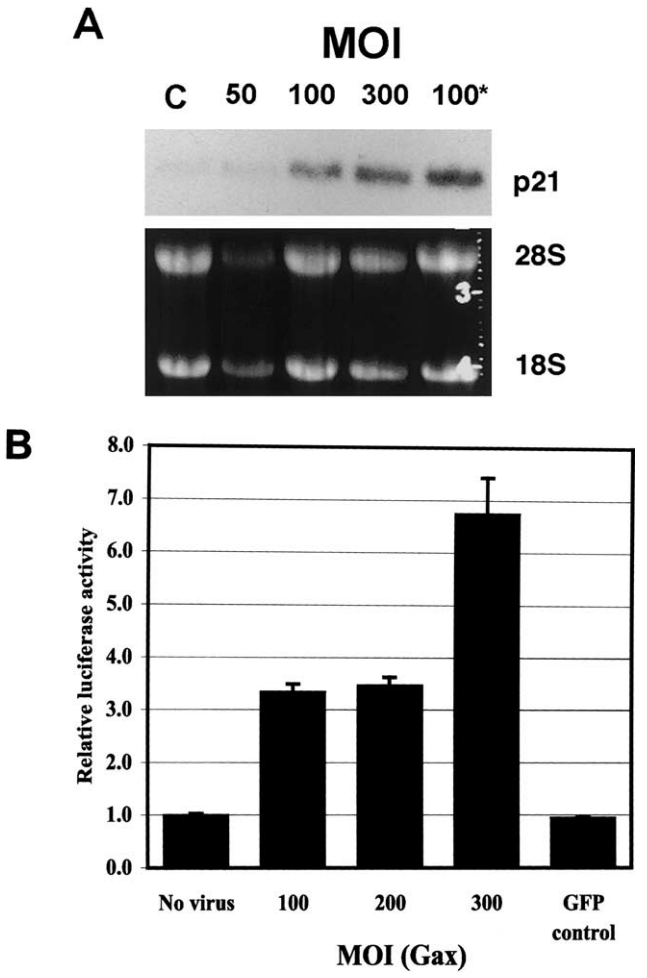

FIG. 5. *Gax* overexpression induces p21 expression. (A) *Gax* expression induces p21 expression in HUVECs. Randomly cycling HUVECs were infected with either Ad.*hGax* at varying MOIs, Ad.*rGax* at MOI = 100(*), or Ad.*GFP* = 300 MOI (C) and then were harvested 24 h later, and Northern blots performed using a p21 probe. (B) Gax expression induces p21 promoter activity. HUVECs were infected with Ad.*rGax* and then transfected with a plasmid containing the p21 promoter driving the firefly Luciferase gene. Luciferase activity was measured 24 h later and normalized to *Renilla* Luciferase activity. Error bars represent standard deviation of 3 wells.

Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype.

In this study, we have reported data strongly suggesting a role for another homeobox gene, the growth arrest homeobox gene *Gax,* in regulating the phenotypic changes that occur in vascular endothelial cells during angiogenesis. Moreover, unlike cell cycle regulators such as p21 or p53, the expression of this gene is relatively restricted to the cardiovascular system [14, 15]. We suspected such a role for *Gax* in endothelial cells during angiogenesis because of its activities in vascular smooth muscle cells, which include G_1 cell cycle arrest [18]; p21 activation [18]; and inhibition of migration towards cytokines and mitogens [19]. We therefore looked for its expression in vascular endothelial cells using RT-PCR, Northern blot, and immunohistochemistry and found that *Gax* is indeed expressed in endothelial cells, both *in vitro* (Fig. 1) and *in vivo* in normal human blood vessels (Fig. 2). Moreover, its expression blocks endothelial cell proliferation, with this inhibition being associated with an upregulation of p21. This upregulation is proportional to the level of expression of *Gax,* and appears to be caused by the activation of the p21 promoter.

Tumor angiogenesis represents a promising new target for anticancer therapy. Given that the most important cell in this process is the vascular endothelial cell, targeting angiogenesis implies targeting vascular endothelial cell processes important to angiogenesis. Specific transcription factors such as *Ets-1* [36] are known to integrate the signals coming from the pathways activated by pro- and antiangiogenic factors and translate these signals to changes in endothelial cell gene expression and phenotype. As such, endothelial cell transcription factors represent both a tool for understanding the phenotypic changes endothelial cells undergo in response to proangiogenic factors secreted by tumor cells that result in angiogenesis and potential targets for the anti-angiogenic therapy of cancer. *Gax* is a homeobox transcription factor originally isolated in vascular smooth muscle cells that has previously been shown to be involved in cardiovascular remodeling [19, 21, 37], inhibiting vascular smooth muscle cell proliferation [18] and migration [19]. We have now shown that *Gax* is also expressed in vascular endothelial cells (Figs. 1 and 2). Moreover, *Gax* inhibits endothelial cell proliferation (Figs. 3 and 4) as well, activating p21 expression (Fig. 5). Of most interest, *Gax* also strongly inhibits tube formation on reconstituted basement membranes (Fig. 6), suggesting that, in addition to its role in inhibiting vascular smooth muscle cell-dependent vascular remodeling processes such as intimal hyperplasia [18, 19], it may also have a role inhibiting vascular remodeling processes that depend mainly on endothelial cells, such as angiogenesis. We therefore conclude that *Gax* may represent an important negative regulator of angiogenesis in vascular endothelial cells, and as such may represent a new molecular tool to understand the transcriptional control of changes in gene expression that occur in endothelial cells during angiogenesis and, more importantly, a potential target for the antiangiogenic therapy of cancer.

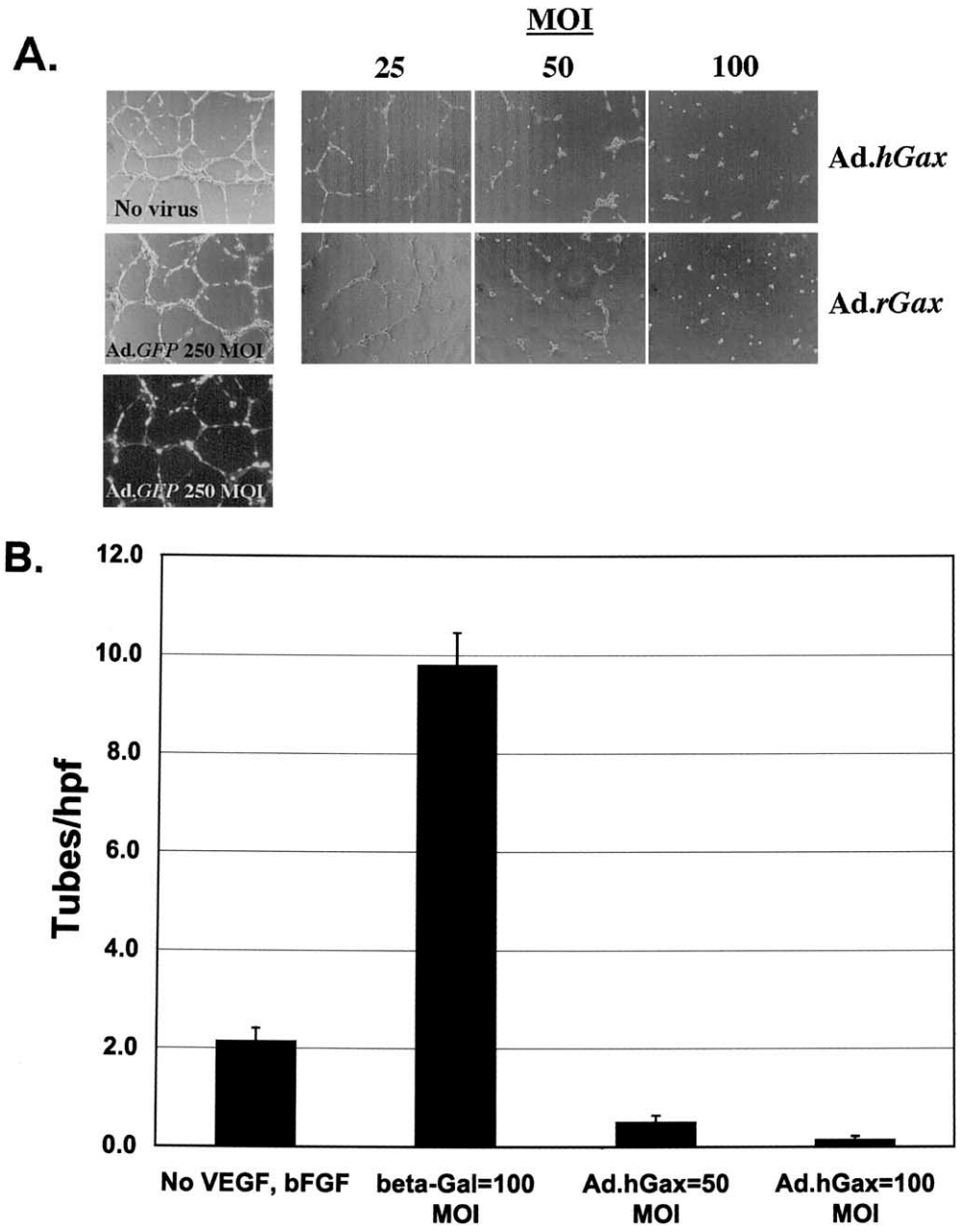

FIG. 6. *Gax* inhibits VEGF-induced endothelial cell tube formation on Matrigel. HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*), or *GFP* (Ad.*GFP*) at the MOI indicated. Eighteen hours later, 5×10^5 cells were plated on Matrigel in 6-well plates and incubated overnight in the presence of serum and 10 ng/ml VEGF. Tube formation was strongly inhibited by both Ad.*hGax* and Ad.*rGax* ($P < 0.05$ at MOI = 25). (A) HUVECs in culture demonstrating the inhibition of tube formation by increasing MOI of Ad.*hGax* and Ad.*rGax* and Ad.β-gal was the control. (B) Tube counts for an experiment in which Ad.*hGax* was used to inhibit endothelial cell tube formation.

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ELSEVIER

Control of Vascular Cell Differentiation by Homeobox Transcription Factors

David H. Gorski* and Kenneth Walsh

Homeobox genes are a family of transcription factors with a highly conserved DNA-binding domain that regulate cell proliferation, differentiation, and migration in many cell types in diverse organisms. These properties are responsible for their critical roles in regulating pattern formation and organogenesis during embryogenesis. The cardiovascular system undergoes extensive remodeling during embryogenesis, and cardiovascular remodeling in the adult is associated with normal physiologic processes such as wound healing and the menstrual cycle, and disease states such as atherosclerosis, tumor-induced angiogenesis, and lymphedema. Aside from their roles in the formation of the embryonic vascular system, homeobox genes recently have been implicated in both physiologic and pathologic processes involving vascular remodeling in the adult, such as arterial restenosis after balloon angioplasty, physiologic and tumor-induced angiogenesis, and lymphangiogenesis. Understanding how homeobox genes regulate the phenotype of smooth muscle and endothelium in the vasculature will improve insight into the molecular mechanisms behind vascular cell differentiation and may suggest therapeutic interventions in human disease.
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Changes in cellular phenotype leading to remodeling in the vascular system occur during normal development and in pathologic states. During embryogenesis, vascular endothelial cell (EC) precursors converge into blood islands, which ultimately develop into the aortic arches and capillary networks that provide oxygen and nutrients to the developing organs and limbs. From this, lymphatic EC precursors bud from embryonic veins to form the lymphatic vascular system. In the adult, examples of changes in vascular cell phenotype leading to vascular remodeling include wound healing and the menstrual cycle, during which both angiogenesis and regression of blood vessels are tightly regulated. Examples of pathologic remodeling include atherosclerosis and arterial restenosis after balloon angioplasty. In both processes, vascular smooth muscle cells (VSMCs) migrate from the media to the intima and proliferate, leading to narrowing of the arterial lumen and the subsequent complications, including hypoxia or even anoxia in downstream tissues (Ross 1993)—quickly in the case of restenosis and slowly in the case of atherosclerosis. In addition, phenotypic changes in vascular ECs leading to vascular remodeling play a critical role in tumor biology because diffusion of oxygen and nutrients limits tumor growth to within 1 mm of a capillary. To overcome this limitation, tumors secrete proangiogenic factors to stimulate the ingrowth of new blood vessels (Folkman 1995), which develop from ECs with an immature phenotype (Eberhard et al. 2000). Similarly, tumors also secrete prolymphangiogenic factors, which allow for the ingrowth of lymphatics and subsequent metastasis to regional lymph nodes (Skobe et al. 2001). Thus, understanding the mechanisms underlying the phenotypic changes that lead to vascular remodeling could produce insights into diseases as diverse as atherosclerosis or restenosis, lymphedema, and cancer.

Although the receptors and signaling pathways activated by growth factors and cytokines have been studied extensively in the vascular system, much less is known about the molecular biology of the downstream transcription factors activated by these pathways to regulate tissue-specific gene expression controlling the growth and differentiation of these cells. Transcription factors represent a common mechanism that can integrate multiple signaling pathways to produce the necessary changes in gene expression and phenotype for vascular cells to perform their functions. Homeobox genes encode a family of transcription factors

containing a common 60-amino-acid DNA-binding motif known as the homeodomain, containing a helix–turn–helix motif similar to that found in prokaryotic regulatory proteins such as Cro, CAP, and the λ repressor in *Escherichia coli* (Scott et al. 1989). They are regulators of cell differentiation, proliferation, and migration in both vertebrates and invertebrates, controlling pattern formation in the embryo and organogenesis, as well as oncogenesis in the adult (Cillo et al. 1999, Ford 1998, Krumlauf 1994). Given these characteristics, homeobox genes are excellent candidates for important roles in the final transcriptional regulation of genes responsible for vascular remodeling and angiogenesis in normal physiology and disease. Recently several homeobox genes have been implicated in the phenotypic changes in vascular cells that lead to intimal hyperplasia, arterial restenosis after angioplasty, angiogenesis, and lymphangiogenesis. It is therefore an opportune time to review briefly what is currently known about homeobox gene expression and activity during vasculogenesis and vascular remodeling in the adult.

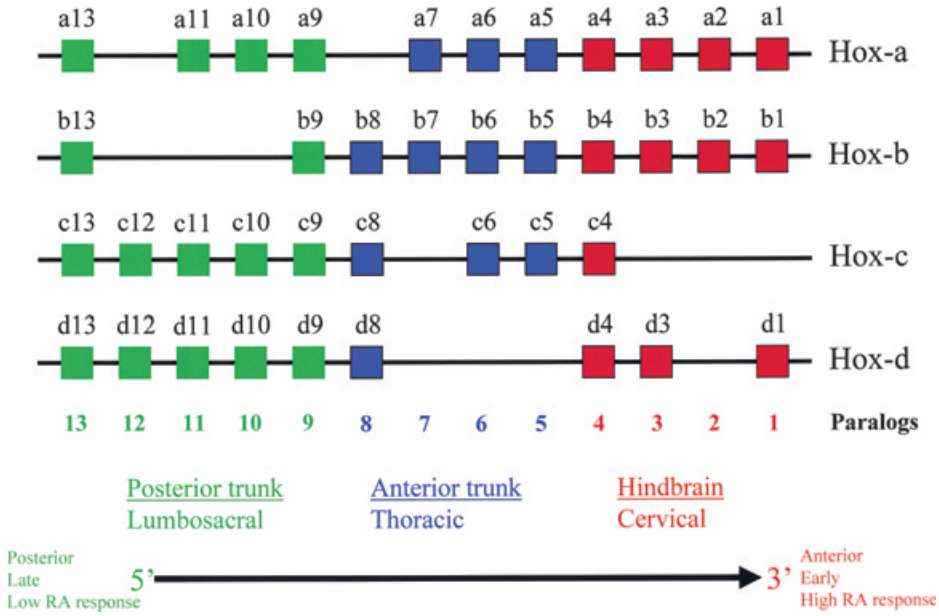

Figure 1. Organization of the HOX clusters. The four HOX clusters in the human and mouse are believed to have evolved through gene duplication. In the human, there are 39 homeobox genes in the HOX clusters (Kosaki et al. 2002). In the mouse, as shown in this figure, the 3′ genes are expressed early in embryogenesis in the more rostral regions of the embryo, whereas the 5′ genes are expressed later in embryogenesis in the caudal regions of the embryo (Cillo et al. 1999). The 3′ rostral genes are highly responsive to retinoic acid (RA), whereas the 5′ caudal genes are less sensitive. Each homeobox gene can have as many as three paralogs in the same position in other HOX clusters. Each HOX cluster is located on a different chromosome. The arrangement of the human HOX clusters, HOX A through D, is nearly identical to the mouse. See text for details.

• Homeobox Gene Expression and Function During Vascular Development

HOX Cluster Genes

In *Drosophilia melanogaster* and vertebrates, many, but not all, homeobox genes are arranged in gene clusters. In mice and humans, there are four unlinked complexes—HOX A through HOX D—that arose from gene duplication (Krumlauf 1994). Because of this, each HOX gene may have as many as three paralogues. The location of each HOX gene in the cluster corresponds to its axial pattern of expression in the developing embryo, with 5′ genes expressed more toward the caudal region and 3′ genes expressed more toward the rostral region (Figure 1), with specific embryonic defects due to knockouts of specific HOX genes occurring in the axial region of their expression. HOX genes have been studied widely with regard to their ability to control pattern formation in the developing embryo. They are powerful regulators of pattern formation, as evidenced by the homeotic mutations (i.e., mutations in which one normal body part is substituted for another normal body part, as in *Antennapedia*).

Several members of the HOX clusters are expressed in the cardiovascular system during embryogenesis, including *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* (Miano et al. 1996). Moreover, there is functional evidence for involvement of HOX genes in vasculogenesis. For example, transgenic mice with null mutations of the *HOXA3* gene die shortly after birth, suffering from defects in the cardiovascular system that include heart-wall malformations, persistent patent ductus arteriosus, and aortic stenosis (Chisaka and Capecchi 1991). In some of these mice, the right carotid artery fails to form, and in all mice the aorta is thin walled and poorly developed. The overall constellation of defects in *HOXA3* null mice is similar to that observed in the human congenital disorder DiGeorge syndrome (Chisaka and Capecchi 1991).

Because paralogous HOX genes have similar DNA-binding domains and axial expression patterns during embryogenesis, it has been hypothesized that they may have overlapping or complementary functions. Thus, targeting one paralogue may not produce an observable phenotype. This has been demonstrated by antisense targeting of the messages for the paralogous HOX 3 group (*HOXA3* and *HOXB3*), which results in the regression of aortic arch 3 in a manner similar to that of arch 2 (Kirby et al. 1997). Similarly, targeting paralogous group 5 genes (*HOXA5*, *HOXB5*, and *HOXC5*) causes the appearance of an additional pharyngeal arch containing a novel and aortic arch artery (Kirby et al. 1997). These observations suggest that paralogues probably have overlapping functions in vascular development and that in at least some cases they can compensate for each other when the function of one is impaired.

Paired-Related Genes

The expression of two genes not located in the HOX clusters—*Prx1* (formerly known as *MHox* or *Phox*) (Cserjesi et al. 1992) and *Prx2* (formerly known as *S8*) (Opstelten et al. 1991)—during embryogenesis suggests that they have an important role in vasculogenesis. In the vascular

system, expression of *Prx1* and *Prx2* is associated with the primary vessel wall and becomes increasingly restricted to the adventitial and outer medial cell layers as development proceeds (Bergwerff et al. 1998). *Prx1* expression colocalizes with procollagen I and fibrillin 2 but not with smooth muscle α actin, whereas *Prx2* expression is highly associated with the developing ductus arteriosus and is one of the earliest markers of its differentiation. Transgenic mice with null mutations *Prx1* and *Prx2* suggest their relative importance in vascular patterning in the embryo. $Prx2^{-/-}$ mutants do not show cardiovascular malformations. In contrast, $Prx1^{-/-}$ mutants display abnormal positioning and awkward curvature of the aortic arch, in addition to a misdirected and elongated ductus arteriosus (Bergwerff et al. 2000). However, $Prx1^{-/-}/Prx2^{-/-}$ double mutants demonstrate a more severe form of these abnormalities, some of them possessing an anomalous retroesophageal right subclavian artery, as well as excessive tortuosity of all great vessels as they run through the mesenchyme, although they do not have cardiac anomalies (Chesterman et al. 2001). Thus, the loss of *Prx2* function exacerbates anomalies due to the loss of *Prx1*, suggesting functional overlap between these two genes in vascular development.

Hex*: An Early Marker of EC Precursors and Regulator of EC and VSMC Differentiation*

Hex is a proline-rich divergent homeobox gene originally isolated from hematopoietic tissues (Crompton et al. 1992). Expressed in a range of hematopoietic progenitor cells and cell lines (Crompton et al. 1992), *Hex* is an early marker of EC precursors and is transiently expressed in the nascent blood islands of the visceral yolk sac and later in embryonic angioblasts and endocardium (Thomas et al. 1998). The *Xexnopus laevis* homologue *XHex* is expressed in vascular ECs throughout the developing vascular network, and its overexpression leads to disruption of vascular structures and an overall increase in EC number (Newman et al. 1997). These observations suggest an important role for *Hex* in the vascular patterning due to the migration and proliferation of EC precursors. In addition, it has been reported recently that *Hex* also is expressed in VSMCs (Sekiguchi et al. 2001). Its expression is upregulated in neointimal VSMCs after balloon injury in the rat, and *Hex* activates the promoter of NMHC-B/SMemb, a nonmuscle-specific isoform of the smooth muscle myosin heavy chain that is expressed during embryonic development of the aorta, declines in the neonate and adult, and is re-induced in vascular lesions.

Given the above experimental observations, it has been assumed that *Hex* promotes the conversion of ECs to the angiogenic phenotype. However, recent evidence does not support that assumption and suggests that the role of *Hex* in controlling vascular phenotype may be more complex than first thought. First, disruption of the *Hex* gene in mouse embryos does not produce a detectable change in the vascular phenotype (Barbera et al. 2000), suggesting that other factors—perhaps the transcription factor *Scl* (Liao et al. 2000)—may compensate for the loss of *Hex* function. Also, it has been reported recently that *Hex* overexpression in human umbilical vein ECs (HUVECs) inhibits in vitro surrogates for angiogenesis, including migration toward vascular endothelial growth factor (VEGF), invasion, proliferation, and tube formation on reconstituted basement membrane (Matrigel) (Nakagawa et al. 2003). In addition, *Hex* was shown to inhibit the expression of angiogenesis-related membrane genes, including those encoding VEGFR-1, VEGFR-2, neuropilin 1, integrin subunit α_V, Tie-1, and Tie-2. It remains to be clarified whether *Hex* inhibits angiogenesis in vivo, but, taken together with previous reports, these observations suggest a complex role for *Hex* in regulating the proliferation and development of the vascular tree and the differentiation of ECs and VSMCs.

Prox1 *and Development of the Lymphatic System*

The lymphatic system is a vascular network of thin-walled capillaries and larger vessels lined by a layer of ECs that drain lymph from the tissue spaces of most organs and return it to the venous system for recirculation. Early in development, primitive lymph sacs develop from endothelial budding from the veins to form the lymphatic system. The homeobox gene *Prox1* has been implicated in the development of the lymphatic system. Originally isolated by its homology to the *Drosophila* gene *prospero* (Oliver et al. 1993), *Prox1* has an expression pattern that suggests a functional role in a variety of tissues, including eye lens, central nervous system, and liver, with null mutations leading to embryonic lethality (Wigle and Oliver 1999). Supporting a role in lymphatic development is the observation that *Prox1* is the earliest marker of lymphatic EC precursors, and in $Prox1^{-/-}$ knockout mice, budding of ECs that give rise to the lymphatic system is arrested at embryonic day 11.5, resulting in mice without lymphatic vasculature (Wigle and Oliver 1999). In contrast, vasculogenesis and angiogenesis are unaffected by the loss of *Prox1* function (Wigle and Oliver 1999, Wigle et al. 2002). In addition, expression of *Prox1* in vascular ECs results in proliferation and a reprogramming of these cells to a lymphatic EC phenotype, inducing expression of lymphatic genes such as *VEGFR-3*, $p57^{kip2}$, and *desmoplakin I/II* and downregulating vascular EC genes such as *STAT6* and *neuropilin 1* (Hong et al. 2002, Petrova et al. 2002). Moreover, this lymphatic reprogramming due to *Prox1* expression occurs only in vascular ECs, although *Prox1* is still able to induce cyclin expression and proliferation in other cell types (Petrova et al. 2002). Together, these data suggest a role for *Prox1* as a general inducer of proliferation and a key regulatory gene in the developing lymphatic system.

• Homeobox Gene Expression and Function in Mature Blood Vessels

Homeobox Gene Expression during VSMC Phenotypic Modulation and Vascular Disease

VSMCs exist within a spectrum of phenotypes ranging from the "contractile" to the "synthetic" state (Ross 1993). Cells in the contractile state are quiescent; do not migrate; are relatively insensitive to mitogens; express contractile proteins, including smooth muscle-specific isoforms of actin and myosin; and are associated with normal vessel wall. Synthetic state cells, on the other hand, are able to migrate; express lower levels of contractile proteins, with higher levels of nonmuscle isoforms of myosin and actin; secrete extracellular matrix components; and generally resemble less-differentiated VSMCs found in fetal blood vessels. Over the last decade, evidence has been accu-

mulating that homeobox genes are involved in regulating the transition between these two phenotypes.

In the adult, several members of the HOX clusters are expressed in the cardiovascular system. Homeobox sequences isolated from adult rat aorta include *HOXA2*, *HOXA4*, *HOXA5*, and *HOXB7*, and *HOXA11* (Gorski et al. 1994, Patel et al. 1992). Other groups have reported the expression of *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* in human adult and fetal aortic smooth muscle (Miano et al. 1996, Patel et al. 1992). Of these, *HOXB7* and *HOXC9* are expressed at markedly higher levels in embryonic VSMCs compared with adult VCMCs, suggesting a role in the proliferation and remodeling that occur during embryogenesis (Miano et al. 1996). In addition, overexpression of *HOXB7* in C3H10T1/2 cells results in increased proliferation; the induction of a VSMC-like morphology; and the expression of early, but not intermediate, VSMC markers. Moreover, *HOXB7* mRNA was detected in human atherosclerotic plaques at a higher level than in normal human arterial media (Bostrom et al. 2000). These observations suggest a role for *HOXB7* and perhaps *HOXC9* in vascular remodeling, either in the expansion of immature VSMCs or the change of vascular myocytes to a more immature phenotype, both of which occur in human vascular diseases, such as atherosclerosis and restenosis after balloon angioplasty.

Gax *and Control of Smooth Muscle Phenotype*

Originally isolated from a rat aorta cDNA library with the use of degenerate oligonuceotide probes directed at the most conserved protein sequence of the *Antennapedia* homeodomain (Gorski et al. 1993a), *Gax* (also known as *Mox-2*) encodes a homeodomain-containing transcription factor whose expression has multiple effects on vascular phenotype. Although its expression is more widespread in the embryo, including all three muscle lineages and brain (Skopicki et al. 1997), *Gax* expression in the adult is more narrowly confined to cardiovascular tissues, including heart, medial smooth muscle cells of arteries, lung, and mesangial cells in the kidney (Gorski et al. 1993a). In VSMCs, *Gax* expression is downregulated rapidly by mitogenic signals such as serum, platelet-derived growth factor (Gorski et al. 1993a), and angiotensin II (Yamashita et al. 1997), and more slowly upregulated by growth arrest signals such as serum deprivation (Gorski et al. 1993a) and C-type natriuretic peptide (Yamashita et al. 1997). Moreover, *Gax* expression is also downregulated in the proliferating VSMCs of the rat carotid artery after balloon injury (Weir et al. 1995). *Gax* expression induces G_0/G_1 cell-cycle arrest and upregulates p21 expression by a p53-independent mechanism, and it is this upregulation of p21 that accounts for its antiproliferative activity (Smith et al. 1997). *Gax* also controls the migration of VSMCs toward chemotactic growth factors through its ability to alter integrin expression, downregulating integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ through the specific suppression of the β_3 and β_5 subunits, both in vitro and in vivo (Witzenbichler et al. 1999). Cell-cycle arrest, which does not by itself suppress VSMC migration, is essential for the antimigratory activity of *Gax*, as *Gax* overexpression has no effect on $p21^{-/-}$ cells. Collectively, these data suggest that *Gax* may function to coordinate vascular cell growth and motility through its ability to regulate integrin expression in a cell-cycle-dependent manner. The ability of *Gax* to induce apoptosis in proliferating VSMCs (Perlman et al. 1998) is consistent with these observations, because integrin signaling is an important regulator of cell viability.

Control of Smooth Muscle Phenotype by Prx

The expression of *Prx1* and *Prx2* cannot be detected in the vasculature of adult rats, but they are upregulated in rat pulmonary arteries in which pulmonary hypertension was induced by the injection of monocrotaline (Jones et al. 2001). Induction of *Prx1* and *Prx2* expression in vitro and in vivo is coincident with induction of the extracellular matrix protein tenascin C, which promotes growth and survival of cultured VSMCs. *Prx1* activates the tenascin-C promoter and induces VSMC proliferation in vitro. Consistent with these observations, *Prx1* is upregulated by angiostatin II and, along with the serum response factor, mediates angiotensin II-induced smooth muscle α-actin expression in VSMCs (Hautmann et al. 1997). Collectively, it appears that *Prx1* and *Prx2* genes have roles both in regulating the proliferation of embryonic VSMCs during the formation of the vascular system and in controlling the change of mature VSMCs to a more immature phenotype that occurs in some vascular diseases.

Homeobox Genes and Postnatal Angiogenesis

Functional evidence for the involvement of HOX cluster genes in the regulation of the angiogenic phenotype comes from the study of the paralogous HOX genes *HOXD3* and *HOXB3*, each of which appears to have distinct and complementary roles in this process. *HOXD3* is expressed at high levels in proliferating ECs induced to form tubes on Matrigel but not in quiescent ECs, and its expression is induced by basic fibroblast growth factor (bFGF) (Boudreau et al. 1997). Functionally, blocking *HOXD3* expression with antisense inhibits the bFGF-stimulated upregulation of integrin $\alpha_V\beta_3$ and urokinase plasminogen activator (uPA) without affecting EC proliferation. In contrast, overexpressing *HOXD3* leads to expression of these genes and a morphologic change to the angiogenic phenotype, resulting in the formation of endotheliomas in vivo. In diabetic mice, *HOXD3* expression is impaired in ECs, as is its upregulation after wounding, suggesting that impaired *HOXD3* expression might be involved in the impaired wound healing observed in diabetics (Uyeno et al. 2001). In addition, the *HOXD3* paralogue, *HOXB3*, has been reported to influence angiogenic behavior in a manner distinct from *HOXD3*. Antisense against *HOXB3* impairs the capillary morphogenesis of dermal microvascular ECs and decreases the phosphorylation of the Eph A2 receptor (Myers et al. 2000). Consistent with this result, constitutive expression of *HOXB3* results in an increase in capillary vascular density and angiogenesis, but does not produce endotheliomas. Taken together, these results suggest overlapping and complementary roles for *HOXB3* and *HOXD3* in angiogenesis, with *HOXD3* promoting the invasive or migratory behavior of ECs in response to angiogenic signals and *HOXB3* promoting capillary morphogenesis of these new vascular sprouts.

In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene—*HOXD10*—

inhibits EC conversion to the angiogenic phenotype. Expression of *HOXD10* is higher in quiescent endothelium as compared with tumor-associated vascular endothelium. Moreover, sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis in the chick chorioallantoic membrane assay in vivo. Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels (Myers et al. 2002) when embedded in Matrigel-containing sponges (Nor et al. 2001) in nude mice. In addition, human ECs overexpressing *HOXD10* express a gene profile consistent with a quiescent, nonangiogenic state, with decreased expression of genes that influence remodeling of the extracellular matrix and cell migration during angiogenesis, such as the uPA receptor and the α_3 and β_4 integrin subunits (Myers et al. 2002). Based on these observations, coupled with the proangiogenic activity of *HOXB3* and *HOXD3*, it has been proposed that the 5′ and 3′ HOX genes have distinct influences on EC behavior, with the more 3′ genes tending to promote the angiogenic phenotype and the more 5′ HOX genes such as *HOXD10* tending to be inhibitory to the angiogenic phenotype and dominant.

The expression of other members of the HOX clusters also have been detected in vascular ECs. One example is *HOXA9EC*, an alternatively spliced variant of HOXA9 whose expression is downregulated by tumor necrosis factor α (TNF-α), which, in addition to its numerous other activities, is proangiogenic (Patel et al. 1999). Also, the expression of several members of the HOX B cluster in HUVECs is regulated by VEGF and tissue plasminogen activator, but not bFGF (Belotti et al. 1998). Because HOX B cluster gene expression does not correlate with the mitogenic state of the cell but rather is altered with the state of cellular differentiation, it has been suggested that these genes are involved in the morphogenic changes associated with the angiogenic phenotype.

Recently it has been reported that *Gax* also is expressed in vascular ECs (Gorski and Leal 2003). As in VSMCs, in ECs, *Gax* expression results in cell-cycle arrest and induces p21 expression and promoter activity. Of note, it also strongly inhibits EC tube formation in response to VEGF on Matrigel (Gorski and Leal 2003) in a manner similar to that of *Hex* (Nakagawa et al. 2003). These additional observations suggest that in addition to its likely role in maintaining VSMCs in the contractile phenotype, *Gax* may also have a role in EC differentiation. Taken together, all of the above observations suggest that *Gax* may be a global inhibitor of vascular cell activation. However, like *Hex* knockout mice (Barbera et al. 2000), mice transgenic for a null mutation in *Gax* have not been reported to show vascular anomalies (Mankoo et al. 1999). Rather, they show skeletal muscle anomalies in the limbs and die shortly after birth from unknown causes. This would tend to suggest that other homeobox factors, such as *Mox-1* (Candia and Wright 1996) or possibly *Pax3* (Stamataki et al. 2001), might compensate for a lack of *Gax*/*Mox-2* expression in the developing cardiovascular system. It would be of great interest to determine whether *Gax* knockout mice demonstrate increased angiogenesis in response to proangiogenic stimuli, but such studies would be difficult because of their very brief life span. Similar studies would also be of interest in *Hex* knockout mice.

Other homeobox genes also are likely to be involved in regulating angiogenesis, whether physiologic or tumor induced. For example, St. Croix et al. (2000) used serial analysis of gene expression to look for expressed sequence tags (ESTs) whose expression is at least 10-fold greater in tumor endothelium compared with normal endothelium. Not surprisingly, many of the ESTs they reported derive from extracellular matrix proteins. However, one EST was similar to the homeobox gene *Dlx-3*, a member of the *Distal-less* family of homeobox genes. This EST was not detectable in the developing corpus luteum, implying a distinction between tumor angiogenesis and physiologic angiogenesis. Interestingly, *Dlx-3* has been implicated in placental function (Beanan and Sargent 2000). Other placental homeobox genes include *Dlx-4*, *Gax*/*Mox-2*, *HB24*, and *Msx2* (Quinn et al. 1997). Given the critical importance of angiogenesis and blood vessel regression in placental function, it is reasonable to predict that some of these genes are involved in vascular remodeling in the placenta. It is also reasonable to postulate that homeobox genes previously demonstrated to be important in inducing proliferation and migration of ECs and EC precursors during angiogenesis—such as *Hex*—also may be important in inducing angiogenesis in the adult vasculature.

• Conclusions

Although much more is known since the last time we reviewed the expression and function of homeobox genes in the vasculature (Gorski et al. 1993b), knowledge of the transcriptional regulation of VSMC and EC phenotype still is not as detailed as is the understanding of the cytokines and growth factors that act on ECs and VSMCs to regulate their phenotype, the receptors these factors activate, and the downstream signaling pathways activated in turn by these receptors. However, a growing number of homeobox genes have been implicated in vascular development in the embryo and vascular remodeling, angiogenesis, and vascular diseases in the adult. Moreover, with the description of *Prox1* (Hong et al. 2002, Petrova et al. 2002), it has become clear that homeobox genes participate in the development of the lymphatic vascular system as well. Given the sheer number of homeobox genes and potential interactions between them and vascular remodeling, it is difficult to generalize too much about the roles of homeobox genes in these processes, some of which are listed in Table 1. It is possible, however, to come to three general conclusions with regard to how homeobox genes regulate vascular remodeling.

1. Pathways controlled by homeobox genes are redundant, especially during embryogenesis. This implies that it is more likely to be the overall pattern of homeobox gene expression rather than any one individual homeobox gene that regulates the phenotype of VSMCs and ECs during angiogenesis and vascular remodeling. The roles of *HOXB3*, *HOXD3*, and *HOXD10* in regulating EC phenotype during angiogenesis represent a good example of this principle. It may be the balance between pro- and antiangiogenic HOX cluster genes that determine whether an EC becomes angiogenic, and different proangiogenic HOX genes may control different stages or aspects of angiogenesis (e.g., *HOXB3* and *HOXD3*). It also can be postulated that *Gax* and *Hex* help to determine this balance. Similarly, in VSMCs, it can be postulated that the balance between *Gax* and *Prx1*/*Prx2* (and possibly *Hex*) plays a major role in

Table 1. Homeobox genes expressed in the cardiovascular system

Cell type	*Gene*	*Function/observation*	*Reference*
VSMC	*Gax (Mox-2)*	Downregulated upon mitogen stimulation and vascular injury Causes G_1 cell-cycle arrest and inhibits VSMC migration Inhibits integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$ expression Induces apoptosis in cycling cells Inhibits restenosis after balloon injury Interacts with *Pax3*	Gorski et al. 1993a, Perlman et al. 1998, Smith et al. 1997, Stamataki et al. 2001, Weir et al. 1995, Witzenbichler et al. 1999, Yamashita et al. 1997
	Hex	Induces expression of immature actin isoform in VSMCs	Sekiguchi et al. 2001
	HOX B7	More highly expressed in fetal VSMCs than in adult VSMCs Induces differentiation of C3H10T1/2 cells into VSMC-like cells	Bostrom et al. 2000, Miano et al. 1996
	HOX C9	More highly expressed in fetal VSMCs than in adult VSMCs	Miano et al. 1996
	HOX A3 and *B3*	*HOX A3* knockout mice have vascular anomalies Blocking *HOX A3* and *B3* causes regression of aortic arch 3	Kirby et al. 1997
	HOX A5, *B5*, and *C5*	Blocking expression causes appearance of additional aortic arch artery	Kirby et al. 1997
	HOX A2, *A4*, *A11*, and *B1*	Isolated from vascular smooth muscle, functions in VSMC unknown	Gorski et al. 1993a and 1994, Patel et al. 1992
	Prx1	Interacts with serum response factor to activate binding Putative role in angiotensin II-mediated smooth-muscle α-actin expression *Prx1*/*Prx2* double-null mutants demonstrate vascular anomalies Activates proliferation and tenascin-C expression	Bergwerff et al. 1998 and 2000, Chesterman et al. 2001, Hautmann et al. 1997, Jones et al. 2001
	Prx2	Widely expressed in embryonic vasculature *Prx1*/*Prx2* double-null mutants demonstrate vascular anomalies	Bergwerff et al. 1998 and 2000, ten Berge et al. 1998
Vascular ECs	*HOXA9EC*	EC specific, function presently unknown Expression modulated by tumor necrosis factor α	Patel et al. 1999
	HOX B cluster	HOX B cluster induced by differentiating factors	Belotti et al. 1998
	HOXB3	Involved in regulating capillary morphogenesis	Myers et al. 2000
	HOXD3	Induces expression of integrin $\alpha_V\beta_3$ Induces angiogenic phenotype in ECs Impaired function associated with impaired wound healing	Boudreau et al. 1997, Uyeno et al. 2001
	HOXD10	Inhibits angiogenesis and changes EC gene expression profile to the nonangiogenic state	Myers et al. 2002
	Dlx-3	Expressed sequence tags with homology to *Dlx-3* expressed at high levels in tumor endothelium Necessary for placental development	Quinn et al. 1997, St. Croix et al. 2000
	Gax (*Mox-2*)	Inhibits in vitro surrogates for angiogenesis May have function in placental–mesenchymal interactions	Gorski and Leal 2003, Quinn et al. 1997 and 2000
	Hex	Early marker of ECs during embryogenesis Expressed throughout the vascular network Overexpression increases EC number in embryos Overexpression blocks EC tube formation on Matrigel	Barbera et al. 2000, Liao et al. 2000, Nakagawa et al. 2003, Newman et al. 1997, Sekiguchi et al. 2001, Thomas et al. 1998
Lymphatic ECs	*Prox1*	Specific to lymphatic ECs Induces expression of lymphatic EC-specific genes Null mutations prevent development of lymphatic system Master regulator of lymphatic vessel formation from embryonic venous system	Hong et al. 2002, Petrova et al. 2002, Wigle and Oliver 1999, Wigle et al. 1999 and 2002

EC, endothelial cell; VSMC, vascular smooth muscle cell.

determining whether VSMCs become contractile or synthetic.

2. Individual homeobox genes may function as master regulatory genes for parts of the vascular system. For instance, although a master regulatory gene controlling development of angioblasts into vascular ECs or VSMCs remains to be identified, *Prox1* represents a very good candidate for such a role in lymphatic endothelium. However, it must be remembered that most homeobox genes controlling vascular phenotype also are expressed in other tissues. Even *Prox1* is expressed in liver and eye lens during embryogenesis. Similarly, *Prx1* is clearly important in skeletal development (ten Berge et al. 1998), and *Gax* is important in skeletal muscle development (Mankoo et al. 1999). This implies that cell-type-specific factors influence the activities of homeobox genes in both ECs and VSMCs and that homeobox genes may be downstream from other, more global, master regulatory genes. Indeed, *Prox1* can only reprogram a vascular EC to take on the phenotype of lymphatic endothelium (Petrova et al. 2002). It cannot so reprogram other cell types.

3. Little is known about how homeobox genes implicated in angiogenesis and vascular remodeling exert their effects at the molecular level. However, it is clear that at least a subset of them appear to function by controlling the differentiation, proliferation, and/or migration of VSMCs and ECs. The mechanism behind these phenotypic changes must be the activation and repression of specific batteries of downstream genes. Because few downstream genes from homeobox genes are known, one of the most fertile areas of research for homeobox gene research is the identification of their downstream targets and the elucidation of the mechanisms by which homeobox genes regulate the expression of these target genes and these target genes in turn lead to the phenotypic changes observed. In the near future, it is likely that cDNA microarray technology will provide an excellent tool for identifying the global changes in gene expression occurring in response to homeobox gene expression in vascular cells.

Given their importance in cell-cycle control, cell migration, and cell adhesion, it is likely that many more homeobox genes will be implicated in the regulation of vascular remodeling and angiogenesis. The identification of the specific homeobox genes involved in these processes, their downstream target genes, and the cell-signaling pathways activated and repressed by homeobox gene expression in vascular ECs and VSMCs will result in a better understanding of the basic cellular mechanisms by which the vascular system is remodeled in response to physiologic signals, tumors, or other stimuli. Such understanding has the potential to lead to the development of therapies that block tumor angiogenesis and lymphatic metastasis, reverse atherosclerosis, prevent restenosis after angioplasty, improve wound healing, and reverse lymphedema.

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The Homeobox Gene *Gax* Inhibits Angiogenesis through Inhibition of Nuclear Factor-κB–Dependent Endothelial Cell Gene Expression

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Abstract

The growth and metastasis of tumors are heavily dependent on angiogenesis, but much of the transcriptional regulation of vascular endothelial cell gene expression responsible for angiogenesis remains to be elucidated. The homeobox gene *Gax* is expressed in vascular endothelial cells and inhibits proliferation and tube formation *in vitro*. We hypothesized that *Gax* is a negative transcriptional regulator of the endothelial cell angiogenic phenotype and studied its regulation and activity in vascular endothelial cells. Several proangiogenic factors caused a rapid down-regulation of *Gax* mRNA in human vascular endothelial cells, as did conditioned media from breast cancer cell lines. In addition, *Gax* expression using a replication-deficient adenoviral vector inhibited human umbilical vein endothelial cell migration toward proangiogenic factors *in vitro* and inhibited angiogenesis *in vivo* in Matrigel plugs. To identify putative downstream targets of *Gax*, we examined changes in global gene expression in endothelial cells due to *Gax* activity. *Gax* expression resulted in changes in global gene expression consistent with a quiescent, nonangiogenic phenotype, with increased expression of cyclin kinase inhibitors and decreased expression of genes implicated in endothelial cell activation and angiogenesis. Further analysis revealed that *Gax* down-regulated numerous nuclear factor-κB (NF-κB) target genes and decreased the binding of NF-κB to its target sequence in electrophoretic mobility shift assays. To our knowledge, *Gax* is the first homeobox gene described that inhibits NF-κB activity in vascular endothelial cells. Because NF-κB has been implicated in endothelial cell activation and angiogenesis, the down-regulation of NF-κB–dependent genes by *Gax* suggests one potential mechanism by which *Gax* inhibits the angiogenic phenotype. (Cancer Res 2005; 65(4): 1414-24)

Introduction

The process of angiogenesis, critical in both normal physiology and in disease states such as cancer and inflammatory diseases, is normally tightly regulated by a balance between pro- and antiangiogenic factors, known as the "angiogenic balance" (1). Tumors manipulate their microenvironment and parasitize the host by secreting factors that induce angiogenesis, tipping the angiogenic balance toward a proangiogenic state. The primary target of tumor-secreted proangiogenic factors is the vascular endothelial cell, which becomes "activated" and undergoes distinct changes in phenotype and gene expression. These changes include activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix (2, 3). Although the endothelial cell receptors and signaling pathways activated by proangiogenic factors such as vascular endothelial growth factor (VEGF; ref. 4) have been extensively studied, less is known about the molecular biology of the downstream transcription factors activated by these factors. Nuclear transcription factors likely integrate these upstream signals, activating and repressing downstream batteries of genes, to produce an angiogenic global gene expression profile, resulting in the angiogenic phenotype. Consequently, understanding the transcriptional mechanisms by which endothelial cells become activated is likely to suggest new therapeutic strategies for inhibiting this process at a very distal point in its signaling cascade, with potential applications in the antiangiogenic therapy of cancer.

Because of their ubiquitous role as regulators of cellular differentiation and body plan formation during embryogenesis, as well as oncogenes and tumor suppressors in various human cancers (5, 6), it is not surprising that homeobox genes have been implicated in regulating the phenotypic changes that endothelial cells undergo during angiogenesis (7). In particular, one diverged homeobox gene, *Gax* (whose mouse homologue is known as *Meox-2*), has several characteristics that suggest that it may play an important role as an inhibitor of the endothelial cell phenotypic changes that occur in response to stimulation by proangiogenic or proinflammatory factors (8–11). Originally isolated from vascular smooth muscle (8) and widely expressed in mesoderm and muscle precursors in the embryo (12, 13), in the adult *Gax* expression is mostly restricted to the cardiovascular system and kidney (8, 13). In vascular smooth muscle cells, *Gax* expression is down-regulated by mitogens and up-regulated by growth arrest signals (8, 14). Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest (10) and inhibits vascular smooth muscle cell migration, modulating integrin expression (11). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (10). Recently, we have reported that *Gax* is also expressed in endothelial cells, in which its expression inhibits endothelial cell proliferation (15) and strongly inhibits VEGF-induced endothelial cell tube formation on reconstituted basement membrane *in vitro* (15), suggesting that *Gax* may be an inhibitor of the activated, angiogenic phenotype.

Until now, we had not identified potential mechanisms by which *Gax* might accomplish its inhibition of endothelial cell activation, other than a general cell cycle arrest due to induction of p21 (10, 15). In this report, we now describe how *Gax* expression is regulated in endothelial cells by proangiogenic and proinflammatory factors and how its expression in endothelial

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cells can block angiogenesis *in vivo*. Finally, we present evidence that *Gax* inhibits nuclear factor-κB (NF-κB) activity in endothelial cells. Given that there is now considerable evidence that activation of NF-κB activity in endothelial cells is proangiogenic (16–22), this interaction between a homeobox gene and NF-κB represents one potential mechanism by which *Gax* expression may inhibit angiogenesis. This interaction, to our knowledge the first described in endothelial cells, may represent a new mechanism by which homeobox genes can interact with intracellular signaling pathways in endothelial cells and thereby inhibit tumor-induced angiogenesis.

Materials and Methods

Cell Lines and Expression Constructs

Human umbilical vein endothelial cells (HUVEC) and EGM-2 medium were obtained from BioWhittaker (Walkersville, MD) and HUVECs cultured according to the manufacturer's instructions. Human microvascular endothelial cells (HMEC)-1 cells were obtained from the Centers for Disease Control and were cultured as described (23). Breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to instructions. Conditioned medium was obtained by incubating them in serum-free medium for 24 hours.

The cloning of the *Gax* cDNA into the mammalian expression vector pCGN to produce pCGN-*Gax* and the construction of replication-deficient adenoviral vectors expressing the rat and human homologues of *Gax* (Ad.h*Gax* and Ad.r*Gax*, respectively) conjugated to the α-hemagglutinin epitope have been described (10). The control replication-deficient adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). An adenoviral construct expressing a form of Akt (T308A, S473A, adenoviral construct designated Ad.DN.Akt) that functions as a dominant negative (24) was kindly provided by Dr. Kenneth Walsh (Boston University, Boston, MA). Expression of *Gax* protein was verified as previously described (13) by Western blot using antihemagglutinin antibody and anti-*Gax* antibodies (not shown). Transfections of HUVECs with pCGN-*Gax* were carried out using Trans-IT Jurkat transfection reagent (Mirus Bio Corporation, Madison, WI) according to a modification of the manufacturer's instructions.

Real-time Quantitative Reverse Transcription–PCR

After treatment as described individually for each experiment, total RNA was isolated from endothelial cells using a spin column with on-column DNase digestion to remove contaminating genomic DNA (RNAeasy, Qiagen, Valencia, CA). First-strand synthesis was done on the total RNA using oligo(dT) primers (SuperScript kit, Invitrogen, Carlsbad, CA), and then message levels for *Gax* and other genes determined by real time quantitative reverse transcription–PCR (RT-PCR) using TaqMan probes (25). Quantitative RT-PCR was carried out using a Cepheid SmartCycler thermocycler, with the associated SmartCycler v.2.0 software used to analyze the data and determine the threshold count (C_t).

Primer and probe sets for each gene were designed using the MacVector 7.2 software package (Accelrys, San Diego, CA). The fluorophore used was 6-carboxyfluorescein (6-FAM), and the quencher was Black Hole Quencher-1 (BHQ-1, Biosearch Technologies, Novato, CA). Sequences of the primers and probes were as follows: *Gax*: 5′-TCA GAA GTC AAC AGC AAA CCC AG-3′ (forward), 5′-CCA GTT CCT TTT CCC GAG-3′ (reverse), 5′-(6-FAM)-TGG TTC CAA AAC AGG CGG ATG-3′-(BHQ1; TaqMan probe), amplicon = 238 bp; E-selectin: 5′-CTC TGA CAG AAG AAG CCA AG-3′ (forward), 5′-ACT TGA GTC CAC TGA AGT CA-3′ (reverse), 5′-(6-FAM)-CCA CGC AGT CCT CAT CTT TTT G-3′ (BHQ1; TaqMan probe), amplicon = 255 bp; vascular cell adhesion molecule-1 (VCAM-1): 5′-ATG ACA TGC TTG AGC CAG G-3′ (forward), 5′-GTG TCT CCT TCT TTG ACA CT-3′ (reverse), 5′-(6-FAM)-CAC TTC CTT TCT GCT TCT TCC AGC-3′ (BHQ1; TaqMan probe), amplicon = 260 bp; intercellular adhesion molecule-1 (ICAM-1): 5′-TAT GGC AAC GAC TCC TTC T-3′ (forward), 5′-CAT TCA GCG TCA CCT TGG-3′ (reverse), 5′-(6-FAM)-CCT TCT GAG ACC TCT GGC TTC G-3′-(BHQ1; TaqMan probe), amplicon = 238 bp; GRO-α: 5′-CAA GAA CAT CCA AAG TGT GAA CG-3′ (forward), 5′-(6-FAM)-AGG AAC AGC CAC CAG TGA GC-3′ (reverse), 5′-CGC CCA AAC CGA AGT CAT AGC-3′-(BHQ-1; TaqMan probe), amplicon=200 bp. Sequences of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe set were 5′-ACA ACT TTG GTA TCG TGG AAG-3′

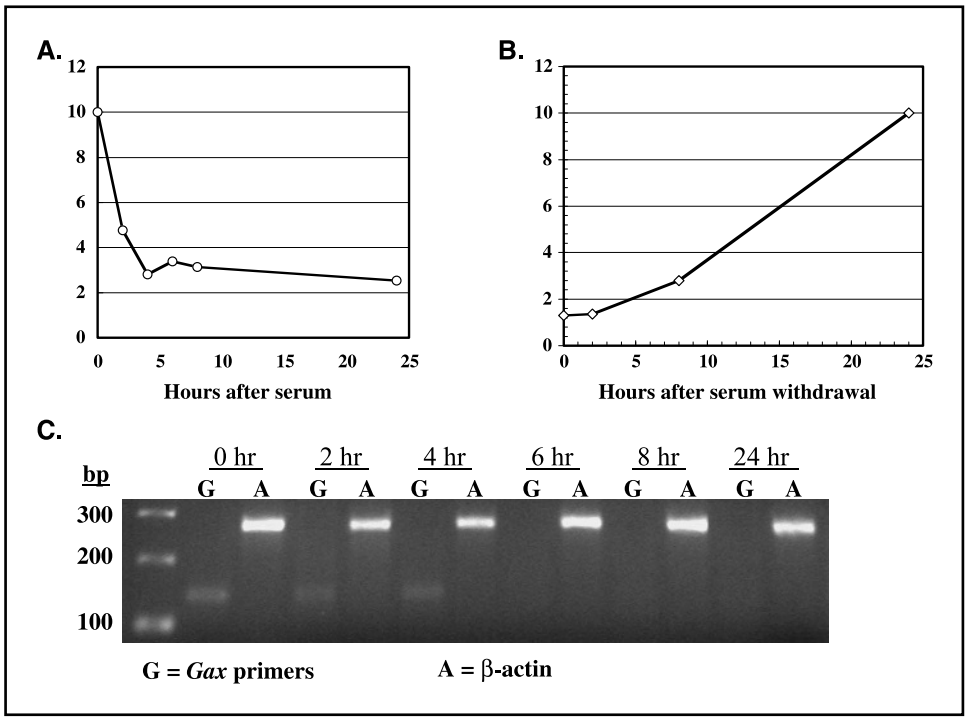

Figure 1. *Gax* expression is down-regulated induced in HUVECs by serum and up-regulated when serum is withdrawn. Using real-time quantitative RT-PCR, *Gax* levels were measured in quiescent HUVECs stimulated with serum and randomly cycling HUVECs placed in low-serum medium. *Gax* levels were normalized to β-actin. For this experiment alone, primers for *Gax* and β-actin previously described were used (15). Similar results were obtained with the primer/probe combination described in Materials and Methods. *A, Gax* is down-regulated by serum. *B, Gax* is up-regulated by serum withdrawal. *C,* PCR gel of the experiment in *A.* Units are arbitrary.

(forward), 5′-CAG ATG AGG CAG GGA TGA TGT TC-3′ (reverse), and 5′ -(6-FAM)-ACC CAG AAG ACT GTG GAT GG-3′ -(BHQ1; TaqMan probe), amplicon = 138 bp. For some experiments (Fig. 1), a set of primers for human *Gax* and β-actin previously described were used (15), along with SYBr Green to monitor the PCR reaction.

Real-time PCR cycles started with an initial 1.5-minute denaturation step at 95°C, followed by 30 to 40 cycles of denaturation at 95°C for 10 seconds; annealing at 50°C (VCAM-1), 52°C (E-selectin, ICAM-1), and 56°C (*Gax*, GAPDH, p21, Gro-α) for 20 seconds; and extension at 72C for 30 seconds. Each sample was run in triplicate and C_t determined for the target gene. For all reactions, negative controls were run with no template present, and random RNA preparations were also subjected to sham quantitative RT-PCR (no reverse transcriptase) to verify lack of genomic DNA contamination. To correct for differences in RNA quality and quantity between samples, target gene levels were normalized to corresponding GAPDH message levels using the $\Delta\Delta C_t$ method (26), as described previously (27, 28).

Migration Assays

Before each experiment, cell culture membranes and flasks were coated with sterile 0.1% gelatin in PBS. HUVECs were infected with adenoviral vectors for 16 hours before 5×10^4 cells per well were plated onto 8.0-μm pore size polycarbonate membrane in 24-well plates. Cells were allowed to attach for 1 hour in EGM-2 medium. Once the cells had attached, the medium in the upper chamber was replaced with low-serum medium [which consisted of EGM-2 + 0.1% fetal bovine serum (FBS) lacking VEGF, basic fibroblast growth factor (bFGF), and epidermal growth factor], and the lower chamber with low-serum medium supplemented with either 50 ng/mL VEGF, 50 ng/mL bFGF, 15 ng/mL tumor necrosis factor (TNF), or 10% FBS. VEGF, bFGF, and TNF-α all obtained from R&D Systems (Minneapolis, MN). After 5 hours, the inserts were washed with PBS and the upper surfaces cleaned with a cotton swab to remove any cells that had not migrated. Finally the cells were fixed with Diff-Quik Stain (Dade Behring, Deerfield, IL) and the inserts washed in PBS and photographed for counting. Cells were counted in five high-powered fields per well. Experiments were repeated at least thrice.

In vivo Angiogenesis Assay

In vivo angiogenesis was assayed by the Matrigel plug assay as described previously (24). These experiments were done under a protocol approved by the Institutional Animal Care and Use Committee at University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School. In brief, cold, low growth factor Matrigel (BD PharMingen, San Diego, CA, 500 μL per mouse) containing bFGF 400 ng/mL (R&D Systems), heparin 10 units/mL (Sigma, St. Louis, MO), and 10^8 plaque-forming units of adenoviral expression vector were injected into the flanks of C57BL/6 mice. After 14 days, the mice were euthanized by CO_2 inhalation, and the plugs carefully removed *en bloc* with surrounding connective tissue. Tissue and plugs were fixed in cold acetone and frozen sections cut at 5 μm. Endogenous peroxidase activity was blocked with dilute H_2O_2. Sections were then blocked with 5% bovine serum albumin (BSA) for 15 minutes, washed with PBS, and then incubated with rat anti-mouse CD31 (PECAM) monoclonal antibody (BD PharMingen) in 1% BSA in PBS overnight. Sections were washed with cold PBS twice and incubated with biotinylated mouse anti-rat IgG1/2a (BD PharMingen) in 1% BSA/PBS. Color was then developed with streptavidin-peroxidase (VectaStain, ABC kit, Vector Laboratories, Burlingame, CA). Sections were counterstained with toluidine blue and vessel counts done as previously described (24, 29). In brief, vascular hotspots were located for each plug near the interface between the plug and surrounding stroma, and blood vessel density estimated as the number of CD31-positive cells per high-powered field. Two sections from each plug were made, at least five high-powered fields per section counted, and the mean ± SE determined for each experimental group. The experiment was repeated twice. Statistical differences were determined by one-way ANOVA using Prism v.4.0 (GraphPad Software, Inc., San Diego, CA), followed by Dunnett's multiple comparison test.

Genome-wide Gene Expression Profiling

We compared global gene expression in control HUVECs transduced with Ad.GFP with that of HUVECs transduced with Ad.r*Gax* or Ad.h*Gax*. Cells were transduced at a multiplicity of infection (MOI) of 100, incubated 24 hours in normal medium, then harvested for total RNA isolation as described above. RNA quality was verified by electrophoresis through formaldehyde-containing agarose gels before use for generating probes. Exogenous *Gax* expression was verified by Western blot (data not shown). Global gene expression was then compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip array set and standard protocols supplied by the manufacturer, with technical assistance from the cDNA Microarray Core Facility of the Cancer Institute of New Jersey. The U133A chip contains probe sets for over 33,000 known genes, along with probes for housekeeping genes for normalization and genomic DNA for evaluation of hybridization quality. Results were analyzed using software provided by the manufacturer and then further analyzed with GeneMAPP (30) to identify signal pathway–dependent changes in gene expression.

Western Blots

Whole cell extracts from TNF-α-treated HUVECs were electrophoresed through 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS plus 5% nonfat dry milk and 0.1% Tween 20 before being incubated with the appropriate dilution of primary antibody (mouse monoclonal anti-VCAM-1 and anti-ICAM-1 and rabbit polyclonal anti-E-selectin, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution. Blots were washed with blocking solution and incubated with secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG; Pierce Biotechnology, Inc., Rockford, IL) and then washed again with blocking solution. Bands were visualized by chemiluminescence using the ECL-Plus reagent (Amersham, Piscataway, NJ).

Flow Cytometry

Cells were harvested after the relevant treatment and resuspended in PBS containing 0.1% sodium azide. Approximately 1×10^5 cells were incubated with FITC-conjugated primary antibody against human E-selectin, VCAM-1, or ICAM-1 (BD Biosciences, San Diego, CA) for 30 minutes on ice. Cells were pelleted and washed twice in PBS/azide before flow analysis on a Beckman-Coulter Cytomics FC500 flow cytometer (Fullerton, CA).

Electrophoretic Mobility Shift Assays

HUVECs were transduced overnight with Ad.GFP or Ad.r*Gax* and then induced with 10 ng/mL TNF-α for 1 hour. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce Biotechnology) and incubated with a biotin end-labeled double-stranded oligonucleotide containing the NF-κB consensus sequence (5′-biotin-AGT TGA GGG GAC TTT CCC AGG C-3′; IDT DNA Technologies, Coralville, IA). The binding reactions, containing 6 to 8 μg of nuclear extract protein, buffer [10 mmol/L Tris (pH 7.5), 50 mmol/L KCl, 1 mmol/L DTT], 1 μg of poly(deoxyinosinic-deoxycytidylic acid), 5 μg BSA, and 20 fmol/L of biotin-labeled DNA, were incubated at room temperature for 20 minutes. Competition reactions were done by adding up to 200-fold excess unlabeled double-stranded NF-κB consensus oligonucleotide to the reaction mixture. Other controls included competition with random oligonucleotide (5′-TAG CAT ATG CTA-3′) and an NF-κB site with a point mutation that abolishes DNA binding (5′-CAC AGT TGA GGC CAC TTT CCC AGG C-3′). Reactions were electrophoresed on a 6% acrylamide gel at 100 V for 1 hour in 0.5× Tris-borate-EDTA buffer and then transferred to positively charged nylon membranes. Biotinylated oligonucleotides were detected with streptavidin-linked horseradish peroxidase and the Pierce LightShift kit (Pierce Biotechnology).

Results

Gax Expression Is Rapidly Down-regulated by Mitogens and Proangiogenic Factors in Endothelial Cells

We first wished to determine how *Gax* expression is regulated by growth factors and proangiogenic peptides in endothelial cells.

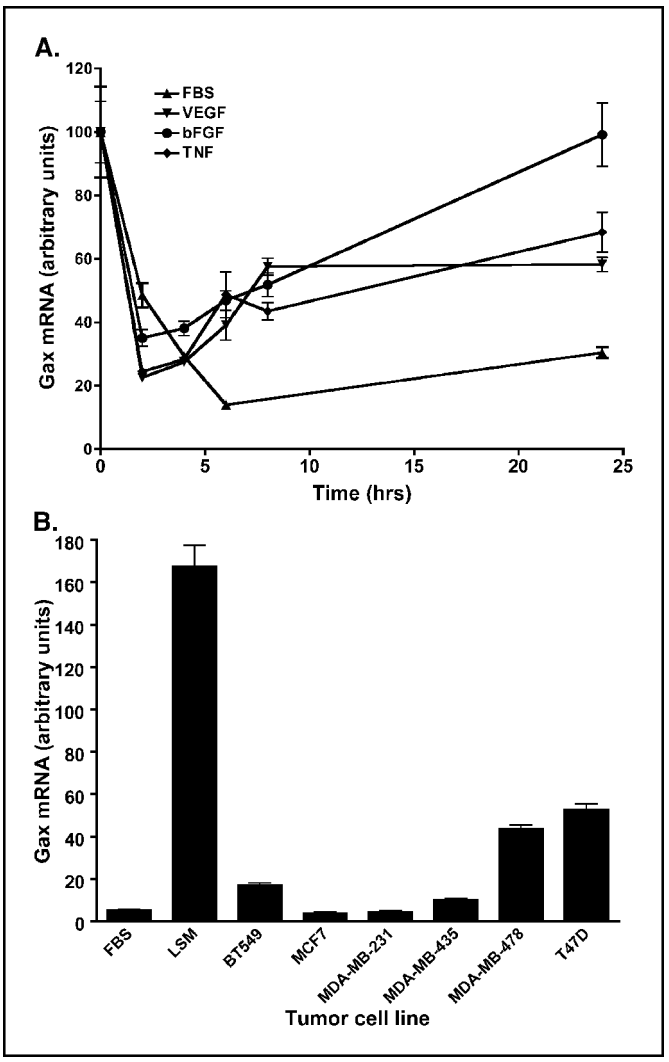

Figure 2. *Gax* down-regulation by mitogens, proinflammatory factors, and tumor-secreted factors. *A,* Mitogens and proangiogenic factors cause rapid down-regulation of Gax expression in endothelial cells. Quiescent HUVECs were treated with either 10% FBS or 10 ng/mL of either $VEGF_{165}$, TNF-α, or bFGF. At various time points, cells were harvested for extraction of total RNA, which was then subjected to quantitative real-time TaqMan RT-PCR with *Gax*- and GAPDH-specific primer/probe sets. (See Materials and Methods for sequences and details.) *B,* down-regulation of *Gax* expression in endothelial cells by conditioned medium from tumor cell lines. Quiescent HUVECs were treated with either low-serum medium, 10% FBS, or 10% conditioned medium from the indicated breast cancer cell lines. Cells were harvested 4 hours after stimulation, total RNA harvested, and real time quantitative RT-PCR done. All *Gax* mRNA levels were normalized to GAPDH expression, and units are arbitrary.

HUVECs made quiescent by incubation for 24 hours in 0.1% FBS were stimulated with 10% FBS plus 5 ng/mL VEGF. *Gax* mRNA was rapidly down-regulated by 5-fold within 4 hours and slowly returned to basal over 24 to 48 hours (Fig. 1*A* and *C*). Conversely, when sparsely plated randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was up-regulated nearly 10-fold within 24 hours (Fig. 1*B*). Quiescent HUVECs were then stimulated with proangiogenic or proinflammatory factors, including bFGF, VEGF, and TNF-α. *Gax* was rapidly down-regulated with a similar time course (Fig. 2*A*). Similar results were observed in HMEC-1 cells (23), an immortalized human microvascular endothelial cell line (data not shown). Finally, conditioned medium from several breast cancer cell lines was used to stimulate quiescent HUVECs for 4 hours. The cell lines varied considerably in their ability to down-regulate *Gax*, but all of them down-regulated *Gax* expression at least 3-fold, and some by as much as 20-fold (Fig. 2*B*), suggesting that tumor-secreted proangiogenic factors also down-regulate *Gax* expression.

Gax Expression Inhibits Endothelial Cell Migration toward Proangiogenic Factors

Migration of endothelial cells through the basement membrane and into the surrounding stroma in response to proangiogenic stimuli is a critical step in tumor-induced angiogenesis. We therefore tested the ability of *Gax* to inhibit endothelial cell migration toward proangiogenic factors. HUVECs were transduced with Ad.r*Gax* or Ad.h*Gax* at varying MOI and incubated overnight. Viable cells (10^5 per well) were plated in six-well plates with inserts containing 8-μm polycarbonate filters and their migration toward angiogenic factor–containing media in the lower chamber

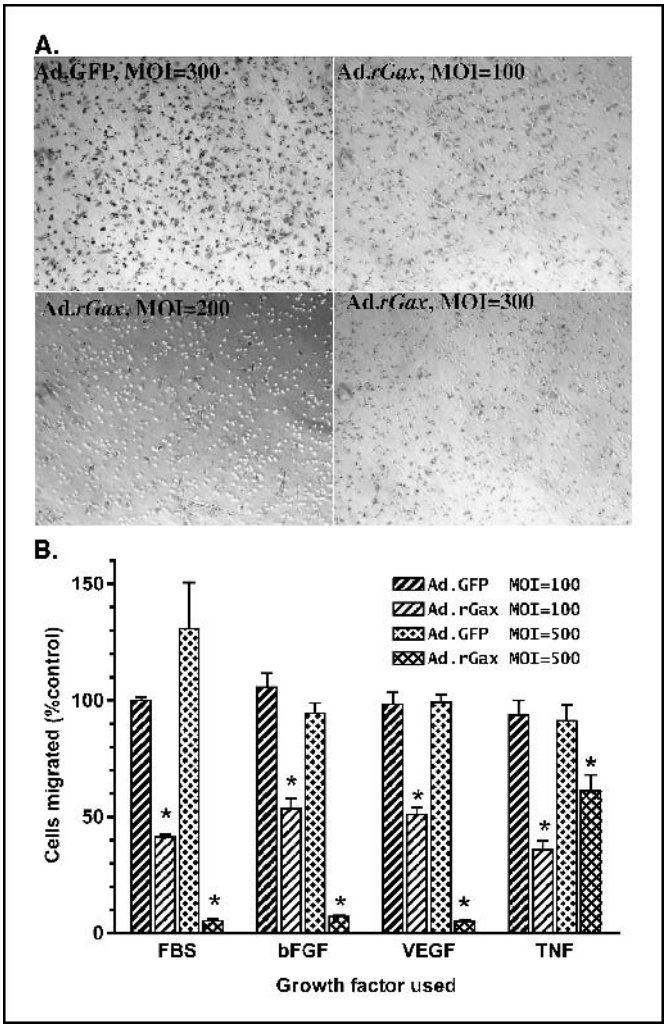

Figure 3. *Gax* inhibits HUVEC migration toward serum. HUVECs were transduced with varying MOIs of either Ad.*GFP* or Ad.r*Gax* and their migration toward various growth factors and proangiogenic factors determined (see Materials and Methods). *Gax* inhibits HUVECs migrating toward (*A*) *FBS*; and (*B*) *FBS*, *bFGF*, $VEGF_{165}$, and *TNF-α*. Results are expressed relative to control HUVECs not transduced with any virus. Results were analyzed by one-way ANOVA; *, $P < 0.01$. Similar results were obtained with Ad.h*Gax* (data not shown).

measured. Ad.r*Gax* strongly inhibited the migration of HUVECs toward serum, VEGF, bFGF, and TNF-α (Fig. 3), as did Ad.h*Gax* (data not shown). Both homologues also inhibited migration of HMEC-1 cells toward bFGF and VEGF (data not shown).

Gax Expression Inhibits *In vivo* Angiogenesis

Matrigel containing proangiogenic factors, when implanted s.c. in mice, can stimulate the ingrowth of blood vessels into the Matrigel plug from the surrounding tissue, allowing *in vivo* tumor cell-free estimates of angiogenesis (24). Moreover, adenoviral vectors diluted in Matrigel implanted as s.c. plugs can serve as reservoirs to transduce endothelial cells invading the plug and drive expression of exogenous genes, producing effects on *in vivo* angiogenesis (31). We therefore used Matrigel plugs to test whether exogenously driven *Gax* expression can inhibit angiogenesis *in vivo*, using methodology previously described (24). Matrigel plugs containing bFGF and either Ad.GFP, Ad.h*Gax*, or Ad.r*Gax* (see Materials and Methods) were injected s.c. into C57BL/6 mice (n = 8 per experimental group). As a positive control for inhibition of angiogenesis *in vivo* by a viral vector, we used an additional adenoviral construct expressing a form of Akt (T308A, S473A, adenoviral construct designated Ad.DN.Akt) that functions in a dominant-negative fashion (24) and has previously been used in the Matrigel plug assay to show that inhibition of Akt signaling inhibits angiogenesis *in vivo* (24). As another control, to verify that adenovirus itself does not significantly alter *in vivo* angiogenesis as measured by this assay, plugs containing only bFGF were also examined. Adenoviral vectors expressing *Gax* expression were observed to inhibit the neovascularization of the plugs with a potency slightly less than what was observed for the Ad.DN-Akt construct (Fig. 4), and the Ad.DN.Akt construct inhibited neovascularization with a potency similar to what has previously been reported (24).

Gax Expression Down-regulates the Expression of NF-κB Target Genes

Next, in order to attempt to identify downstream targets and signaling pathways regulated by *Gax* expression, we determined differences in global gene expression between control HUVECs infected with Ad.GFP with HUVECs infected with Ad.r*Gax* or Ad.h*Gax*. Cells were infected at an MOI = 100, incubated 24 hours

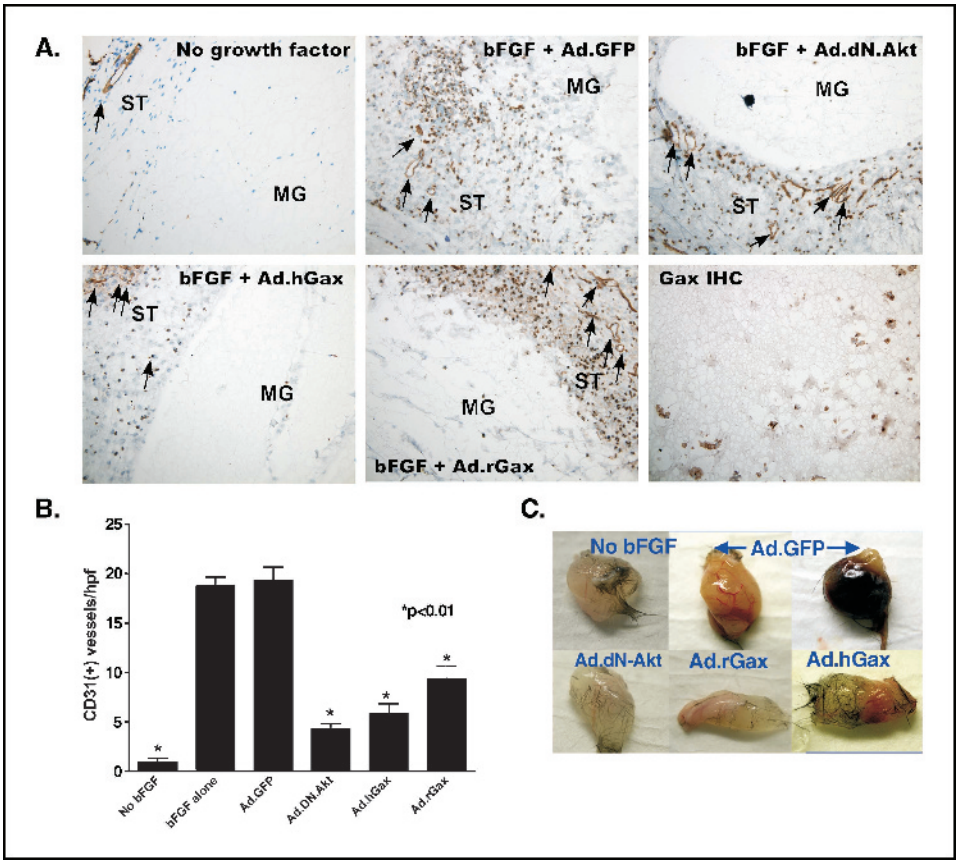

Figure 4. Effect of *Gax* expression on angiogenesis in Matrigel plugs. Matrigel plugs (500 μL each) containing 400 ng/mL bFGF and the indicated viral constructs at 10^8 plaque-forming units per plug were implanted s.c. in the flanks of C57BL6 mice. Plugs were harvested after 14 days incubation for immunohistochemistry using CD31 antibodies and determination of CD31-postitive cells per high powered (400x) field (see *Materials and Methods* and *Results* for details). *MG,* Matrigel plug; *ST,* stroma surrounding the plug. *Arrows,* examples of CD31-positive blood vessels. *A, Gax* inhibits in vivo angiogenesis. Plugs with either no growth factor or bFGF plus Ad.GFP, Ad.dN.Akt, Ad.h*Gax*, or Ad.r*Gax* were implanted into the flanks of C57BL/6 mice (see Materials and Methods for details and concentrations). After 14 days, the mice were euthanized and the plugs harvested for immunohistochemistry with CD31. Immunohistochemistry using anti-*Gax* antibodies according to previously described methods (13) was done on a representative plug into which Ad.rGax had been introduced to show that the construct is transducing the cells within the plug (*lower right hand corner*). *B,* vessel counts. *Columns,* means; *bars,* SE. Statistical differences determined with one-way ANOVA; $P < 0.0001$ for the overall. The vessel counts were statistically significantly different from control (Ad.GFP group) for Ad.DN.Akt ($P = 0.013$), Ad.h*Gax* ($P = 0.008$), and Ad.r*Gax* ($P = 0.028$). *C,* gross photographs of selected plugs. Note the hemorrhage into one of the Ad.GFP plugs and the lack of vessels on the capsule of the Ad.*Gax* and Ad.dN.Akt plugs.

Table 1. Genes regulated by *Gax* expression

Genbank no.	Gene	Function	Fold change	*P*
Up-regulated Genes				
L37882	Frizzled homologue 2 (FZD2)	Signal transduction	30.4	<0.0001
NM_025151	Rab coupling protein (RCP)	Signal transduction	30.1	0.0026
AI678679	Bone morphogenetic protein receptor, type IA (BMPR1A, ALK3)	Signal transduction	27.9	0.0015
N74607	Aquaporin 3 (AQP3)	Transport	19.9	0.0011
AI983115	Class I cytokine receptor	Signal transduction	12.1	<0.0001
NM_002276	Keratin 19 (KRT19)	Structural protein	9.2	<0.0001
NM_004727	Solute carrier family 24 member 1 (SLC24A1)	Ion transport	9.2	0.0007
NM_004585	Retinoic acid receptor responder (tazarotene induced) 3	Cell growth inhibition	8.5	0.0077
K01228	Proα 1 (I) chain of type I procollagen	Structural protein	6.4	0.0001
NM_000361	*Thrombomodulin (THBD)*	*Coagulation*	*5.5*	*0.0006*
NM_006931	Solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	Biosynthesis/metabolism	5.3	0.0000
NM_000850	Glutathione *S*-transferase M4 (GSTM4)	Biosynthesis/metabolism	4.9	0.0009
NM_002064	Glutaredoxin (thioltransferase; GLRX)	Biosynthesis/metabolism	4.9	0.0001
AF162769	Thioltransferase	Biosynthesis/metabolism	4.6	<0.0001
NM_002166	*Inhibitor of DNA binding 2 (ID2)*	*Transcriptional regulation*	*4.6*	*<0.0001*
NM_017436	α1,4-galactosyltransferase; 4-*N*-acetylglucosaminyltransferase (A14GALT)	Biosynthesis/metabolism	4.3	0.0003
NM_005904	MAD (mothers against decapentaplegic) homologue 7 (MADH7)	Signal transduction	4.3	0.0006
NM_000170	Glycine dehydrogenase (GLDC)	Biosynthesis/metabolism	4.0	0.0003
NM_002222	Inositol 1,4,5-triphosphate receptor, type 1 (ITPR1)	Signal transduction	4.0	0.0000
NM_000229	Lecithin-cholesterol acyltransferase (LCAT)	Biosynthesis/metabolism	4.0	0.0002
M25915	Complement cytolysis inhibitor (CLI)	Complement activation	3.7	<0.0001
AF326591	Fenestrated-endothelial linked structure protein (FELS)	Structural protein	3.7	<0.0001
NM_001666	GTPase activating protein 4 (ARHGAP4)	Signal transduction	3.7	<0.0001
NM_006456	Sialyltransferase (STHM)	Biosynthesis/metabolism	3.7	0.0001
NM_000050	Argininosuccinate synthetase (ASS)	Biosynthesis/metabolism	3.7	<0.0001
AF035620	BRCA1-associated protein 2 (BRAP2)	Biosynthesis/metabolism	3.5	0.0002
M25915	Cytolysis inhibitor (CLI)	Complement activation	3.5	<0.0001
NM_006736	Heat shock protein, neuronal DNAJ-like 1 (HSJ1)	Stress response	3.5	<0.0001
NM_000693	Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)	Biosynthesis/metabolism	3.5	<0.0001
NM_000213	*Integrin subunit, 4 (ITGB4)*	*Cell adhesion*	*3.5*	*0.0001*
NM_003043	Solute carrier family 6, member 6 (SLC6A6)	Transport	3.5	0.0001
AF010126	Breast cancer-specific protein 1 (BCSG1)	Unknown	3.2	0.0002
NM_005345	Heat shock 70kD protein 1A (HSPA1A)	Stress response	3.2	<0.0001
NM_006254	Protein kinase C, δ (PRKCD)	Signal transduction	3.0	0.0001
NM_000603	Nitric oxide synthase 3 (endothelial cell; NOS3)	Biosynthesis/metabolism	3.0	<0.0001
U20498	Cyclin-dependent kinase inhibitor p19INK4D	Cell cycle	2.5	0.0004
NM_001147	*Angiopoietin 2 (ANGPT2)*	*Cell growth/chemotaxis*	*2.2*	*0.0023*
N33167	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	Cell cycle	2.1	0.0065
Down-regulated genes				
NM_002167	*Inhibitor of DNA binding 3 (ID3)*	*Transcriptional regulation*	*−2.0*	*0.0081*
D13889	*Inhibitor of DNA binding 1 (ID1)*	*Transcriptional regulation*	*−2.1*	*0.0052*
NM_001546	*Inhibitor of DNA binding 4 (ID4)*	*Transcriptional regulation*	*−2.1*	*0.0056*
M60278	***Heparin-binding epidermal growth factor–like growth factor***	***Cell growth/chemotaxis***	***−2.1***	***0.0056***
NM_001955	***Endothelin 1 (EDN1)***	***Cell growth/chemotaxis***	***−2.5***	***0.0007***
NM_000201	***Intercellular adhesion molecule 1 (ICAM1)***	***Signal transduction***	***−2.5***	***0.0059***
NM_004995	*Matrix metalloproteinase 14*	*Proteolysis*	*−2.7*	*0.0002*
NM_002006	*Fibroblast growth factor 2 (basic; FGF2)*	*Cell growth/chemotaxis*	*−2.8*	*0.0244*
NM_004428	*Ephrin-A1 (EFNA1)*	*Cell growth/chemotaxis*	*−3.0*	*0.0042*
AF021834	Tissue factor pathway inhibitor β (TFPIβ)	Coagulation	−3.0	0.0007

(Continued on the following page)

Table 1. Genes regulated by *Gax* expression (Cont'd)

Genbank no.	Gene	Function	Fold change	*P*
NM_016931	NADPH oxidase 4 (NOX4)	Biosynthesis/metabolism	−3.2	0.0029
NM_021106	Regulator of G-protein signaling 3 (RGS3)	Signal transduction	−3.5	0.0059
NM_002130	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble; HMGCS1)	Biosynthesis/metabolism	−3.5	0.0008
NM_001146	*Angiopoietin 1 (ANGPT1)*	*Cell growth/chemotaxis*	*−3.9*	*0.0012*
NM_005658	***TNF receptor–associated factor 1***	***Signal transduction***	***−4.0***	***0.0086***
NM_001721	BMX nonreceptor tyrosine kinase (BMX), mRNA	Signal transduction	−4.3	0.0007
NM_006226	Phospholipase C, epsilon (PLCE)	Signal transduction	−4.3	0.0012
NM_006823	Protein kinase (cyclic AMP–dependent, catalytic) inhibitor α (PKIA)	Signal transduction	−4.3	0.0002
NM_002425	*Matrix metalloproteinase 10*	*Proteolysis*	*−4.4*	*0.0002*
NM_016315	CED-6 protein (CED-6)	Vesicle-mediated transport	−4.6	0.0059
NM_000600	***Interleukin 6 (IFN, β 2; IL6)***	***Cell growth/chemotaxis***	***−4.6***	***0.0020***
M68874	Phosphatidylcholine 2-acylhydrolase (cPLA2)	Signal transduction	−4.9	0.0007
U58111	***Vascular endothelial growth factor C (VEGF-C)***	***Cell growth/chemotaxis***	***−5.3***	***0.0020***
NM_003326	**TNF (ligand) superfamily, member 4 (TNFSF4)**	**Signal transduction**	**−5.7**	**0.0021**
AB040875	Cystine-glutamate exchanger	Biosynthesis/metabolism	−6.1	0.0012
NM_006290	***TNF-α-induced protein 3 (A20, TNFAIP3)***	***Apoptosis***	***−6.4***	***0.0009***
S69738	**Monocyte chemotactic protein human (MCP-1)**	**Cell growth/chemotaxis**	**−6.5**	**0.0303**
NM_012242	Dickkopf homologue 1 (DKK1)	Signal transduction	−8.0	0.0002
NM_002852	**Pentaxin-related gene, rapidly induced by IL-1 β (PTX3)**	**Immune response**	**−9.2**	**0.0142**
L07555	**Early activation antigen CD69**	**Signal transduction**	**−10.6**	**0.0042**
NM_001078	***Vascular cell adhesion molecule 1 (VCAM1)***	***Cell adhesion***	***−13.0***	***0.0303***
NM_002993	**Granulocyte chemotactic protein 2**	**Cell growth/chemotaxis**	**−17.5**	**0.0059**
NM_012252	Transcription factor endothelial cell	Transcriptional regulation	−18.5	0.0302
NM_000963	Prostaglandin-endoperoxide synthase 2	Biosynthesis/metabolism	−26.0	0.0303
NM_001993	***Coagulation factor III (thromboplastin, tissue factor)***	***Coagulation***	***−39.4***	***0.0022***
NM_000450	***E-selectin (SELE)***	***Cell adhesion***	***−62.6***	***0.0142***
M57731	***Chemokine (C-X-C motif) ligand 2 (CXCL2, GRO-)***	***Cell growth/chemotaxis***	***−79.6***	***0.0007***
NM_002090	***Chemokine (C-X-C motif) ligand 3 (CXCL3)***	***Cell growth/chemotaxis***	***−119.9***	***0.0029***
NM_000584	***Interleukin 8 (IL-8)***	***Immune response***	***−181.3***	***0.0142***
NM_004591	***Chemokine (C-C motif) ligand 20 (CCL20)***	***Cell growth/chemotaxis***	***−237.6***	***0.0376***
N-M_001511	***Melanoma growth stimulating activity, α/GRO-1/GRO-α (CXCL1)***	***Cell growth/chemotaxis***	***−238.9***	***0.0059***

NOTE: Boldface, genes induced by NF-κB activity; italicized, genes involved in regulating angiogenesis.

in normal media, then harvested for total RNA isolation. Global gene expression was compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip array set (see Materials and Methods). We observed 127 probe sets corresponding to known genes showing greater than 2-fold up-regulation and 115 showing greater than 2-fold down-regulation. Differences in gene expression between controls and *Gax*-transduced cells ranged from up-regulation by approximately 30-fold to down-regulation by 239-fold. This pattern was similar in endothelial cells transduced by Ad.h*Gax*, although the magnitude of changes in gene expression tended to be smaller (data not shown). We report here only probe sets that represent known genes that were either up- or down-regulated by at least 2.5-fold, with the addition of a few genes regulated <2.5-fold selected because they are either involved in angiogenesis, regulated by NF-κB, or both (Table 1).

Consistent with the hypothesis that *Gax* inhibits endothelial cell activation, *Gax* strongly down-regulated several CXC chemokines (Table 1). Most strongly down-regulated of all was GRO-α (CXCL1), a CXC chemokine and a growth factor for melanoma that has also been implicated in promoting angiogenesis (32). *Gax* also down-regulated cell adhesion molecules known to be up-regulated in endothelial cells during activation and angiogenesis, including VCAM-1, ICAM-1, and E-selectin (33), all of whose down-regulation we have confirmed using real time quantitative RT-PCR, Western blot, and flow cytometry (Fig. 5). Moreover, *Gax* inhibited both the basal and TNF-α-induced up-regulation of ICAM-1, VCAM-1, and E-selectin proteins (Fig. 5*C* and *D*, and not shown). The pattern of down-regulation of these adhesion molecules, which are normally up-regulated during endothelial cell activation and angiogenesis, coupled with the down-regulation of CXC chemokines, suggested the inhibition of genes normally induced by TNF-α, which in turn suggested the possibility that *Gax* may inhibit NF-κB activity. Indeed, when our data was analyzed using GeneMAPP (30) to look for patterns of signal-dependent gene regulation, numerous NF-κB-dependent genes were identified (Table 1). Western blot analysis showed no difference between untransduced endothelial cells and cells transduced with Ad.GFP in either the

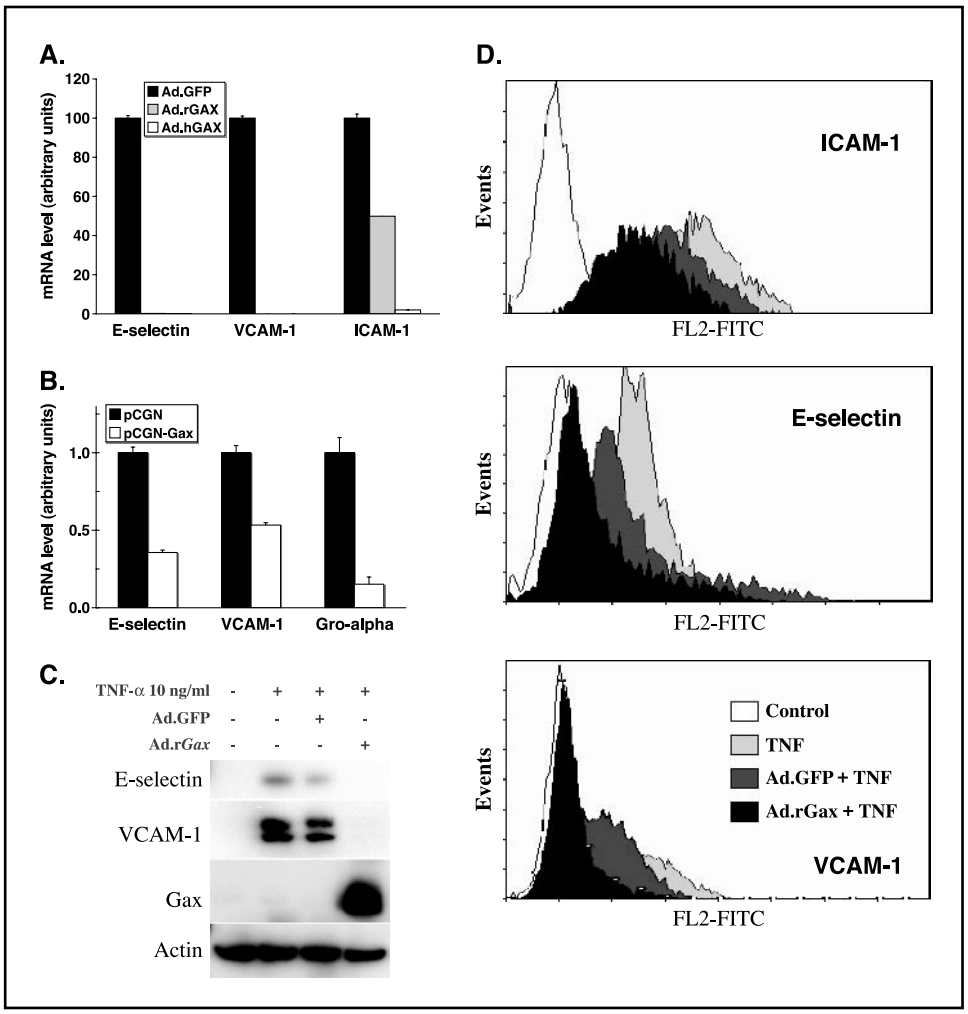

Figure 5. Effect of *Gax* expression on the level of E-selectin, VCAM-1, and ICAM-1. *A, Gax* down-regulates cell adhesion molecule mRNAs in HUVECs. HUVECs were transduced with Ad.GFP, Ad.h*Gax*, or Ad.r*Gax*, incubated for 24 hours in normal growth medium, then harvested for total RNA isolation. Total RNA was then subjected to quantitative real time RT-PCR using TaqMan primers and probes specific for each gene and the results normalized to GAPDH. A very strong down-regulation of E-selectin, VCAM-1, and ICAM-1 message level was observed. *B, Gax* down-regulates NF-κB-dependent genes using nonviral transduction. To rule out artifacts from GFP expression, HUVECs were transfected with pCGN-Gax or pCGN empty vector and then incubated overnight in growth medium. Cells were then harvested for total RNA, which was subjected to real time quantitative RT-PCR as described in Materials and Methods. Despite the lower transfection efficiency of liposomal-mediated methods, a strong down-regulation of NF-κB-dependent genes was observed compared with the empty vector. Units are arbitrary for (*A*) and (*B, C*). *C, Gax* down-regulates HUVEC expression of cell adhesion molecules. HUVECs were transduced with Ad.r*Gax* or Ad.GFP and then incubated overnight, after which they were stimulated with 10 ng/mL TNF-α for 4 hours. Cells were harvested for total protein and subjected to Western blot with appropriate antibodies. Expression of *Gax* from the adenoviral vector was verified by Western blot with antibodies against *Gax* as previously described (13). *Gax* also down-regulated ICAM-1 (not shown). *D, Gax* down-regulates cell surface expression of ICAM-1, E-selectin, and VCAM-1. HUVECs transduced overnight with either Ad.GFP or Ad.r*Gax* at an MOI = 100 were stimulated with TNF-α 10 ng/mL for 4 hours and then harvested for flow cytometry using appropriate antibodies (see Materials and Methods). Ad.r*Gax* blocked the expression of VCAM-1, E-selectin, and ICAM-1.

TNF-α-induced expression of VCAM-1 or E-selectin (Fig. 5*C*) or the basal level of VCAM-1, ICAM-1, or E-selectin protein (not shown), and only slight differences by flow cytometry (Fig. 5*D*), suggesting that our result is not an artifact of our use of Ad.GFP as a control in the initial gene expression profiling experiment. Further supporting this conclusion is our observation by quantitative real time RT-PCR that (1) there was no difference between untransduced HUVECs and those transduced with Ad.GFP in the expression of E-selectin, ICAM-1, VCAM-1, Gro-α, VEGF-C, bFGF, $p21^{CIP1/WAF1}$, and a variety of other genes identified in Table 1 as being regulated by *Gax* (data not shown); and (2) that the same result was obtained for Gro-α, E-selectin, and VCAM-1 using nonviral means of transducing the HUVECs in which no GFP-containing vectors were used (Fig. 5*B*).

In contrast, the genes up-regulated by *Gax* did not fall into any signal-dependent patterns as striking as the genes down-regulated by *Gax* (Table 1). However, there were still results that might suggest specific pathways up-regulated by *Gax*. First, there was a strong up-regulation of ALK3 (bone morphogenetic receptor 1a; 34). Although it is known that ALK1 activates endothelial cells through a SMAD1/5 pathway and ALK5 inhibits endothelial cell activation through a SMAD2/3 pathway (35), it is not known what role ALK3 plays in regulating endothelial cell phenotype. Second, we observed the up-regulation of three CDK inhibitors, $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (10, 36, 37), consistent with a role in promoting cell cycle arrest and the quiescent phenotype. Finally, *Frizzled-2* was strongly up-regulated. Little is known about the potential role of *Frizzled* receptors and Wnt signaling in regulating

postnatal angiogenesis, although *Frizzled-2* is expressed in endothelial cells (38) and there is evidence suggesting Wnt signaling inhibits endothelial cell proliferation (39).

Gax Expression Blocks NF-κB Binding to its Consensus DNA-Binding Sequence

Given that NF-κB activity has been implicated in the changes in phenotype and gene expression endothelial cells undergo during angiogenesis caused by VEGF, TNF-α, and other factors (16–22), we wished to confirm our findings from gene expression profiling that *Gax* inhibits NF-κB activity in endothelial cells. We therefore did electrophoretic mobility shift assays with a probe containing an NF-κB consensus sequence (40) utilizing nuclear extracts from HUVECs transduced with either Ad.r*Gax* or the control adenoviral vector Ad.GFP. *Gax* expression in HUVECs markedly reduced specific binding to NF-κB consensus sequence by nuclear extracts compared with what was observed in controls (Fig. 6*A*), implying that *Gax* expression interferes with the binding of NF-κB to its consensus sequence. Unlabeled double-stranded NF-κB consensus oligonucleotide competed with labeled probe for binding (Fig. 6*B*), and random oligonucleotide and an NF-κB site with a point mutation that abolishes DNA binding (see Materials and Methods for sequences) failed to compete with the probe-specific band (data not shown).

Discussion

Interactions between tumors and their surrounding stroma, particularly the ability of tumors to induce angiogenesis, are critical to tumor progression and metastasis (41). At the endothelial cell level, the process of angiogenesis involves complex temporally coordinated changes in phenotype and global gene expression in response to alterations in the balance between pro- and antiangiogenic factors (2, 3). The stimuli for these changes are communicated from the surface of endothelial cells to the nucleus through multiple overlapping signaling pathways. The peptide factors and the receptors they bind to that activate these pathways have been the subject of intense study over the last decade, because the importance of aberrant endothelial cell activation and angiogenesis to the pathogenesis of not just cancer, but of other diverse human diseases, such as atherosclerosis, diabetic retinopathy, psoriasis, and others, has become more apparent (42). Because blocking aberrant angiogenesis has the potential to be an effective strategy to treat or prevent cancer and other angiogenesis-dependent diseases, understanding how downstream transcription factors integrate upstream signals from pro- and antiangiogenic factors to alter global gene expression and produce the activated, angiogenic phenotype, has become increasingly important.

Homeobox genes represent a class of transcription factors that, given their ubiquitous roles in controlling body plan formation during embryogenesis, organogenesis, cell proliferation and differentiation, and numerous other important cellular processes (5, 7), might be expected to be involved in either promoting or inhibiting the conversion of quiescent, unactivated endothelial cells to the activated, angiogenic phenotype. Indeed, several homeobox genes (*HOXA9EC*, *HOXB3*, *HOXB5*, *HOXD3*, *HOXD10*, and *Hex*) have already been implicated in this process (7, 43). We postulated that at least one additional homeobox gene, *Gax*, is also likely to play an important role in regulating endothelial cell angiogenesis. Consistent with its regulation in vascular smooth muscle cells, in endothelial cells, *Gax* is rapidly down-regulated by serum, proangiogenic, and proinflammatory factors (Figs. 1 and 2), and is able to inhibit endothelial cell migration *in vitro* (Fig. 3) and angiogenesis *in vivo* (Fig. 4). These observations led us to examine the mechanism by which *Gax* inhibits endothelial cell activation by examining global changes in gene expression due to *Gax*. In addition to observing that *Gax* up-regulates cyclin kinase inhibitors and down-regulates a number of proangiogenic factors, we also found that *Gax* inhibits the expression of NF-κB target

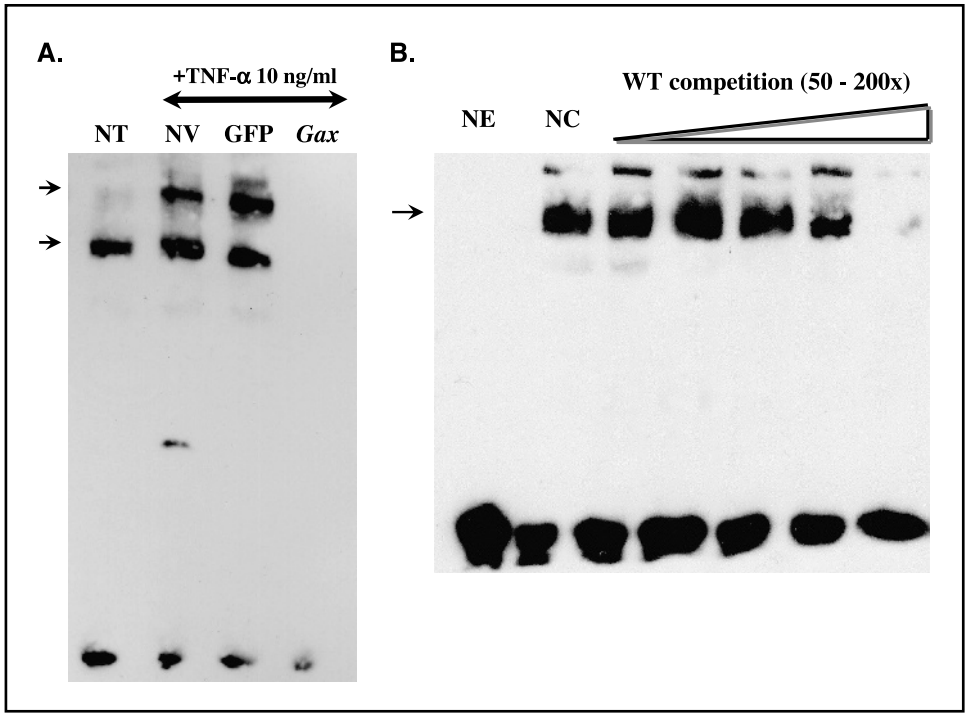

Figure 6. *Gax* expression inhibits NF-κB activity. *A, Gax* blocks NF-κB binding to its consensus sequence. HUVECs were infected with adenovirus containing GFP or r*Gax*, incubated overnight in EGM-2, and then induced with 10 ng/mL TNF-α for 1 hour. Controls were not induced with TNF-α. Nuclear extracts were prepared and incubated with biotinylated oligonucleotides containing the consensus NF-αB binding site (see Materials and Methods). *B,* control electrophoretic mobility shift assay. Excess unlabeled wild-type NF-κB oligonucleotide competes with NF-κB probe. Random oligonucleotide and an NF-κB site with a point mutation that abolishes DNA binding (see Materials and Methods for sequences) failed to compete with the probe-specific band (data not shown). Moreover, *Gax* expression did not affect binding to an unrelated probe (*Oct-1*, data not shown). *Arrows,* NF-κB specific bands, and *bands at the bottom of the gels* represent unbound probe. *NT,* no treatment with TNF-α; *NV,* no virus; *NE,* no nuclear extract; *NC,* no unlabeled competitor; and *WT,* wild-type.

genes (Table 1). Consistent with expression profiling data, *Gax* inhibits the binding of NF-κB to its consensus sequence (Fig. 6).

Several lines of evidence implicate NF-κB activity in regulating endothelial cell phenotype during inflammation and angiogenesis (16–19). For example, proangiogenic factors such as VEGF (33), TNF-α (44), and platelet-activating factor (17) can all activate NF-κB signaling and activity in endothelial cells. In addition, inhibition of NF-κB activity blocks tube formation *in vitro* on Matrigel (22), and pharmacologic inhibition of NF-κB activity suppresses retinal neovascularization *in vivo* in mice (45). Similarly, $\alpha_5\beta_1$-mediated adhesion to fibronectin also activates NF-κB signaling and is important for angiogenesis, and inhibition of NF-κB signaling inhibits bFGF-induced angiogenesis (16). One other potential mechanism by which NF-κB signaling may promote angiogenesis is through an autocrine effect, whereby activation of NF-κB induces expression of proangiogenic factors such as VEGF, as has been reported for platelet-activating factor–induced angiogenesis (17). Alternatively, the involvement of NF-κB in activating endothelial cell survival pathways is also likely to be important for sustaining angiogenesis (46).

Although NF-κB or IκB activity can regulate the expression of homeobox genes (47), there have been few reports of functional interactions between homeodomain-containing proteins and NF-κB or IκB proteins. The first such interaction reported was between IκBα and *HOXB7*, in which IκBα was reported to bind through its ankyrin repeats to the *HOXB7* protein and thus potentiate *HOXB7*-dependent gene expression (48). In contrast, the POU factor *Oct-1* can compete with NF-κB for binding to a specific binding site in the TNF-α promoter because its consensus sequence is close to the NF-κB consensus sequence (49). In addition, at least one interaction has been described in which a homeobox gene directly inhibits NF-κB-dependent gene expression, an interaction in which *Cdx2* blocks activation of the cyclooxygenase-2 promoter by binding p65/RelA (50). It remains to be elucidated if *Gax* inhibits NF-κB-dependent gene expression by a similar mechanism. Regardless of the mechanism, however, this report represents to our knowledge the first description of a homeobox gene that not only inhibits the phenotypic changes that occur in endothelial cells in response to proangiogenic factors but also inhibits NF-κB–dependent gene expression in vascular endothelial cells while doing so. These properties suggest *Gax* as a potential important transcriptional inhibitor of endothelial cell activation and thus a potential target for the antiangiogenic therapy of cancer or other angiogenesis-dependent diseases. In addition, understanding the actions of *Gax* on downstream target genes, signals that activate or repress *Gax* expression, and how *Gax* regulates NF-κB activity in endothelial cells is likely to lead to a better understanding of the mechanisms of tumor-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of cancer.

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The homeobox gene *Gax* activates p21$^{WAF1/CIP1}$ expression in vascular endothelial cells through direct interaction with upstream AT-rich sequences

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RUNNING TITLE: ***Gax activates p21$^{WAF1/CIP1}$***

KEY WORDS: *Gax*, homeobox genes, angiogenesis, vascular endothelium, cell cycle, transcription factors

ABSTRACT

Tumors secrete proangiogenic factors to induce the ingrowth of blood vessels from the surrounding stroma, the end targets of which are vascular endothelial cells. *Gax*, a homeobox gene, has been shown to inhibit angiogenesis and induce p21$^{WAF1/CIP1}$ expression in vascular endothelial cells. In order to elucidate further the mechanism through which *Gax* activates p21$^{WAF1/CIP1}$ expression, we constructed *Gax* cDNAs with deletions of either the N-terminal domain, the homeodomain, or the C-terminal domain, and then assessed these constructs for their ability to activate p21$^{WAF1/CIP1}$. We found that there was an absolute requirement for the homeodomain. Deleting the C-terminal domain decreased, but did not abolish, transactivation of the p21$^{WAF1/CIP1}$ promoter, while deleting the N-terminal domain or the *opa* repeat in the N-terminal domain did abolish transactivation. Next, we performed chromatin immunoprecipitation and found approximately 15 kb upstream of the p21$^{WAF1/CIP1}$ ATG an ATTA-containing *Gax* binding site (designated A6) with a sequence similar to that of other homeodomain binding sites. Gax was able to bind to A6 in a homeodomain-dependent manner and thereby activate the expression of a reporter gene coupled to this sequence, and this activation was abolished by mutating specific residues in this sequence. We conclude that *Gax* activates p21$^{WAF1/CIP1}$ at least in part through an upstream AT-rich sequence that appears to act as an enhancer. Given the multiple biological activities of *Gax* in regulating EC function, identification of a putative *Gax* binding site will allow study of how *Gax* activates or represses other downstream targets to inhibit angiogenesis.

INTRODUCTION

Angiogenesis is critical to the growth, invasion, and metastasis of human tumors. Without a blood supply, tumors are limited by the diffusion of oxygen and nutrients to a size of approximately 1 mm in diameter (5). Key to the process of angiogenesis is the vascular endothelial cell (EC) (21). In health ECs respond to a balance between pro- and antiangiogenic factors secreted by various cell types to maintain blood vessel homeostasis, and it is where this balance lies that determines whether ECs become angiogenic in response to normal physiologic needs in processes as diverse as wound repair, the menstrual cycle, embryogenesis, and organogenesis (4, 30). During carcinogenesis, tumors hijack this process by secreting proangiogenic factors in order to supply themselves with oxygen and nutrients necessary for their continued growth, a transition known as the "angiogenic switch" (4, 30). Because targeting angiogenesis has become a promising avenue of treatment for malignancies (20), understanding the transcriptional regulation of the angiogenic phenotype in ECs leading to activation and angiogenesis has become particularly important.

Vascular ECs respond to extracellular proangiogenic signals by invading through the basement membrane, sprouting, proliferating, forming tubes, and finally attracting pericytes to support the structure of the neovasculature (2, 3, 62). Although the extracellular signals and the immediate downstream signaling pathways activated by pro- and antiangiogenic factors have been topics of intense study (17-19, 61), less is known about the detailed downstream transcriptional regulation that leads to the upregulation and downregulation of batteries of genes necessary to cause ECs to change to the angiogenic phenotype. Because of their ubiquitous nature and their importance in regulating body plan formation, organogenesis, cell proliferation and migration, and even tumor formation, we considered it likely that homeodomain proteins

(15, 22, 27, 37, 41) are involved in the transcriptional regulation of EC angiogenic phenotype, and indeed several homeobox genes have now been so implicated (6-8, 13, 16, 23, 39, 43-45, 47, 54, 67). For example, *HOXD3* expression activates the angiogenic phenotype in vascular ECs through the activation of urine plasminogen activator and integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ (6, 7). In contrast, *HOXD10* activates a gene expression program that inhibits angiogenesis (44), and recently it has been reported that *HOXA5*, a HOX cluster gene implicated in regulating p53 expression in human breast cancer (14, 51, 52), inhibits angiogenesis through the downregulation of VEGFR1 and ephrin A1 (54).

Recently, we described a homeobox gene *Gax* (also known as *Meox-2*) (10, 12, 25, 38), whose expression strongly influences EC phenotype. Originally isolated from a rat aorta cDNA library (25, 26) and studied in vascular smooth muscle (25, 40, 49, 60, 66), in the adult *Gax* is expressed primarily in the cardiovascular system and highly vascularized tissues, such as kidney and lung (25, 59). Pointing to a role in regulating vascular smooth muscle proliferation and phenotype was its pattern of regulation, in which mitogenic signals resulted in a rapid downregulation of *Gax* expression, while growth arrest signals induced a slower upregulation (25). Given its association with mesoderm (11, 12), we looked for the expression of *Gax* in vascular ECs and found that it had a similar pattern of expression as it did in vascular smooth muscle (23, 47), and that mitogenic and proangiogenic signals rapidly downregulated its expression (47). *Gax* has also been shown to inhibit nuclear factor-κB (NF-κB) signaling in vascular ECs (47). VEGF stimulates NF-κB activity, resulting in the translocation of p65 into the nucleus and the expression of a number of proangiogenic genes (1, 29), and inhibition of NF-κB activity is antiangiogenic in several systems (34, 36, 55, 56, 58, 68). Moreover, *Gax* also inhibits

angiogenesis in both *in vitro* and *in vivo* models (23, 47), although recently it has been reported that *Gax* can positively regulate angiogenesis in brain vasculature (67).

Another potentially important mechanism through which *Gax* could inhibit tumor-induced angiogenesis, is the inhibition of cell-cycle progression by activating the cyclin kinase inhibitor p21$^{WAF1/CIP1}$. In vascular smooth muscle cells, *Gax* expression strongly activates p21$^{WAF1/CIP1}$ expression by trans-activating the p21$^{WAF1/CIP1}$ promoter, leading to G_0/G_1 cell cycle arrest, an effect that is p53-independent (60). In vascular ECs, *Gax* also trans-activates the p21$^{WAF1/CIP1}$ promoter, leading to inhibition of proliferation (23). However, the sequences in the p21$^{WAF1/CIP1}$ promoter and the likely mechanisms through which *Gax* accomplishes cell cycle arrest in vascular ECs have not yet been elucidated. In this study, we dissected the Gax protein to identify the homeodomain and polyhistidine (*opa*) repeat as being essential for DNA binding and trans-activation, respectively. We also looked for upstream sequences through which *Gax* activates p21$^{WAF1/CIP1}$ expression in ECs using chromatin immunoprecipitation and identified an upstream sequence that activates its expression. These findings suggest that a major component of the mechanism by which inhibits angiogenesis is its ability to induce cell cycle arrest in ECs by directly activating p21$^{WAF1/CIP1}$ expression and thus inhibiting the early, proliferative stage of angiogenesis. *Gax* may thus represent a potentially promising target for the antiangiogenic therapy of human tumors.

MATERIALS AND METHODS

Cells and cell culture

Human umbilical vein endothelial cells (HUVECs) and EGM-2 medium were obtained from BioWhittaker (Walkersville, MD) and HUVECs cultured according to the manufacturer's instructions.

Flow cytometry/cell cycle analysis

Flow cytometry and cell cycle analysis were performed using HUVECs as previously described (60). In brief, cells were infected with adenoviral vector Ad.*hGax* or control Ad.*GFP* overnight after reaching 50-60% confluence. The cells were then harvested and resuspended in cold PBS. Approximately 1 x 10^6 cells were fixed with 3 ml of -20°C cold absolute ethanol for 1 hour at 4°C, washed twice, and then incubated with 1 ml of 50 μg/ml propidium iodide (PI) staining solution supplemented with 50 μl of 10 μg/ml of RNase A for 3 hours at 4°C. Cells were pelleted and washed twice in PBS before flow analysis on a Beckman-Coulter Cytomics FC500 flow cytometer (Fullerton, CA).

Gene expression assays

Plasmid, adenoviral, and retroviral constructs. *Gax* has three main domains that are likely to have different functions in DNA-binding and transcriptional activation or repression (25). Consequently, *Gax* deletions were constructed using polymerase chain reaction. First, a full length (aa1-aa303) human *Gax* (hugax) cDNA clone (25, 38) was isolated from HUVEC total RNA using reverse transcription polymerase chain reaction (RT-PCR) with appropriate primers. Next, the N-terminal fragment of *Gax*, hugax-NT (aa1-aa187), hugaxΔCT (aa1-aa245) and an N-terminal deletion fragment of *Gax*, hugaxΔNT (aa188-aa303), were generated using PCR with appropriate primers. Constructs in which either the *Gax* homeodomain was deleted (hugaxΔHD, Δaa188-aa245) or the *Gax* CAX (*opa*) repeat was deleted (hugaxΔCAX) were produced by overlap PCR. All cDNA deletion constructs were designed to contain *Eco*RI and *Xho*I restrict enzyme sites and were initially cloned into the pCR-Blunt II-TOPO vector (Invitrogen, CA), after which they were inserted into pcDNA3.1 expression vector (Invitrogen, CA) using the BamHI-EcoRI sites in frame with a Flag tag at the N-terminal end of the peptides. Mammalian

retroviruses were similarly constructed as derivatives of LZRSpBMN-Z in which the lacZ gene had been excised to make LZRSΔ (35). N-terminal Flag-tagged human *Gax* and its deletion constructs with were digested with *BamH*I and *Xho*I enzymes and then cloned into the BamHI-XhoI sites of LZRSΔ (35). All PCR-amplified cDNA constructs were sequenced completely, and expression of protein was verified by Western blot after transient transfection or retrovirus infection.

The cloning of the *Gax* cDNA into the mammalian expression vector pCGN to produce pCGN-*Gax* and the construction of replication-deficient adenoviral vectors expressing the rat and human homologs of *Gax* (Ad.*hGax* and Ad.*rGax*, respectively) conjugated to the α-hemagluttinin (HA) epitope have been described (60). The control replication-deficient adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). Finally, the p21$^{WAF1/CIP1}$ promoter-Luciferase plasmid was also obtained from Dr. Kenneth Walsh (Boston University). It contains approximately 2 kb of upstream sequence from the transcriptional start site, sequence that encompasses the p53 binding sites, and is the same construct used in (60).

Transfections.

Transfections of HUVECs and HMEC-1 cells were carried out using Trans-IT® Jurkat Transfection Reagent (Mirus Bio Corporation, Madison, WI) according to a modification of the manufacturer's instructions using the amounts of plasmids as described for each individual experiment. In general, a 1 μl:1 μg ratio of transfection reagent to plasmid was used, and cells were exposed to reagent-DNA complexes for 1-3 hours, depending upon the experiment, after which they were incubated 24 hours and then harvested for experiments.

Western blots

Whole cell extracts or cytoplasmic extracts from treated HUVECs were electrophoresed through 12% SDS-polyacrylamide gels and transferred to polyvinylidene diflouride membranes. The membranes were blocked with PBS plus 5% nonfat dry milk and 0.1% Tween 20 before being incubated with the appropriate dilution of primary antibody (mouse monoclonal anti-Flag, mouse monoclonal anti-α-tubulin, mouse monoclonal anti-p21$^{WAF1/CIP1}$, mouse monoclonal anti-cyclin D1, and mouse monoclonal anti-p53,Sigma MO) in blocking solution. Blots were washed with blocking solution and incubated with secondary antibody (goat anti-mouse IgG Pierce Biotechnology, Inc., Rockford, IL) and then washed again with blocking solution. Bands were visualized by chemiluminescence using the ECL-Plus reagent (Amersham, Piscataway, NJ).

Confocal microscopy

HUVECs were seeded on coverslips, allowed to attach overnight, and then transfected with pCDNA3.1-Flag-hugax and its truncates. Cells were then rinsed with PBS and fixed in 4% paraformaldehyde for 10 min. at room temperature, followed by incubation with methanol for 20 min. on ice. After three such washes, the cells were permeabilized with 1% Triton X-100-PBS for 10 min, and further blocked with 5% goat serum for one hour. Immunostaining was then carried out using a monoclonal antibody to the Flag tag (Sigma, MO) at 4°C overnight and the protein of interest visualized using Alexa Fluor488-labeled anti-mouse IgG for two hours at room temperature (Molecular Probes, OR). Cell nuclei were stained with TO-PRO-3 iodide (Molecular Probes, OR). Fluorescence was analyzed by using Nikon C1 digital eclipse confocal microscope system.

Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation experiments utilizing HUVECs expressing *Gax* and *Gax* truncates were carried out as follows. HUVECs in 150 mm dishes were infected with either

158 LZRSΔ vector, LZRSΔ-flag-hugax, and LZRSΔ-hugax for 2 days, and then incubated in fresh
159 EGM-2 media (Cambrex, MD) overnight. Next, formaledehyde (37%) was added directly to
160 tissue culture media to a final concentration of 1% with gentle shaking for 10 minutes at room
161 temperature to crosslink the protein-DNA complexes, after which a final concentration of 0.125
162 M of glycine was added and the cells further incubated for 5 minutes to stop the crosslinking
163 reaction. The cells were then rinsed twice with cold 1X PBS containing protease inhibitors
164 (PMSF and protease inhibitor cocktails); harvested into conical tubes by gentle scraping; and
165 pelleted by centrifugation at 2000 rpm for 4 minutes at 4°C, after which the pellets were washed
166 once with 1X PBS containing protease inhibitors. The cell pellets were resuspended in 300 µl of
167 cell lysis buffer containing protease inhibitors and incubated on ice for 10 minutes to release the
168 nuclei. Following that, the nuclei were pelleted by centrifugation for 5 minutes at 5000 rpm at
169 4°C. Lysis buffer containing protease inhibitors (300 µl) was added to the pelleted nuclei, and
170 the mixture incubated on ice for 10 minutes to lyse the nuclei and release the chromatin.

171 Chromatin samples were sonicated on ice to an average length of 600 bp and then
172 pelleted by centrifugation for 10 minutes at 14,000 rpm at 4°C. The supernatant was transferred
173 to a new tube and precleared by adding 30 µl of blocked protein A beads (Sigma, MO) to 1 ml of
174 IP supernatant with gentle shaking for 30 min at 4°C. After preclearing, the supernatant was
175 recovered after pelleting the beads by centrifugation. Target protein-DNA complexes were
176 immunoprecipitated by adding 30 µl of blocked anti-FLAG antibody beads (Sigma, St. Louis,
177 MO) to each sample, followed by incubation at 4° C overnight. To the IP input control was
178 added 10 µl of blocked protein G beads. The antibody-protein-DNA complex samples were
179 collected by centrifugation for 2 min 14,000 rpm at 4°C. Pellets were washed with 1X dialysis
180 buffer twice and 1 ml of IP wash buffer four times at room temperature. Following washing of

the beads, immunoprecipitated antibody-protein-DNA complexes were eluted with 250 µl of freshly prepared IP elution buffer (1%SDS, 0.1M NaHCO3). To reverse crosslinking, 5 M NaCl was added to each combined eluate to a final concentration of 0.3 M, followed by heating at 65°C for 5 hours. Finally, DNA was purified and eluted into 50 µl of H_2O using a DNA gel extraction purification kit (Qiagen, Valencia, CA). To detect the enrichment of chromatin sequences in the immunoprecipitate due to Gax binding in the chromatin upstream of p21$^{CIP1/WAF1}$, we chose ChIP primers to amplify approximately 200 bp fragments at 1-2 kb intervals beginning near the p21$^{WAF1/CIP1}$ start codon and continuing upstream 15 kb. Sequences for the primer sets used for PCR in the initial ChIP assay are listed in Table 1. Approximately 0.5 to 1 µl of sample was used per PCR reaction. Purified DNA from each ChIP assay (0.5 to 1 ul) was subjected to PCR, under the following conditions: initial denaturation at 94° C for 2 min; then 35 cycles at 94 °C for 30 seconds (denaturation), 60° C for 30 seconds (annealing), 72° C for 45 seconds (extension). PCR products were subjected to gel electrophoresis through 2% agarose gels. After initial identification of a putative Gax DNA binding site, we verified that this binding depended upon the homeodomain by performing ChIP on HUVECs transduced with the *Gax* deletion constructs, including LZRSΔ-flag-hugax-NT, LZRSΔ-hugaxΔCT, LZRSΔ-hugaxΔNT, and LZRSΔhugaxΔHD.

Promoter assays

To verify regulation of p21$^{WAF1/CIP1}$ transcription by genomic fragment A in which positive binding for Gax protein in the ChIP assay was observed, 1.5 kb fragments containing p21$^{WAF1/CIP1}$ ChIP-A and p21$^{WAF1/CIP1}$ ChIP-C were cloned and inserted into pGL3. Co-transfection assays of reporter plasmid DNA and pCDNA3.1-flag-hugax and pCDNA3.1-flag-hugaxΔHD were performed the same as described above in order to determine which domains of

the Gax protein are important for regulation of p21$^{WAF1/CIP1}$ transcription. We also searched the p21$^{WAF1/CIP1}$ genomic DNA for additional AT-rich sites resembling the one found in ChIP fragment A and found there was another ATTA-core site in the p21$^{WAF1/CIP1}$ primary promoter region contained in our p21-Luciferase reporter construct (60). luciferase reporter plasmid inserted fragment p21$^{WAF1/CIP1}$. In addition, a construct in which ChIP-C was modified by deletion of the ATTA-core AT-rich site was made by overlap PCR to eliminate the AT-rich site in the core p21$^{WAF1/CIP1}$ promoter.

Promoter activities were measured using constructs with the relevant regulatory sequences placed upstream of Luciferase, using a plasmid containing Luciferase from *Renilla reniformus* under the control of the SV40 promoter (pRL-SV) cotransfected as a normalization control for transfection efficiency. Firefly and *Renilla* Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI), and firefly Luciferase activity from the p21$^{WAF1/CIP1}$-Luciferase promoter construct normalized to constitutive *Renilla* Luciferase activity from the pRL-SV plasmid. For each experiment, HUVECS at approximately 80% confluence in 6-well plates were transfected with differing amounts of plasmid as described in individual experiments. HUVECs were then incubated with transfection reagents for three hours, and then refreshed with fresh EBM (Cambrex,MD) and supplements (Cambrex,MD) overnight. The firefly luciferase and *Renilla* luciferase activity were then measured as described above. In luciferase reporter assays of regulation of *Gax* and its truncates on the expression of p21$^{WAF1/CIP1}$, the volume of 0.5 ug of pGL2-p21 $^{WAF1/CIP1}$ reporter plasmid DNA was fixed, and the dosage of pCDNA3.1-flag-hugax and its truncates plasmid DNA was adjusted according to the cotransfection ratio, with pCDNA3.1-Flag vector used to equalize the total plasmid DNA transfected for each well.

Quantitative reverse transcriptase real time polymerase chain reaction (QRT-PCR)

After ChIP as described individually for each experiment was carried out, the resuspended chromatin immunoprecipitate and flow-through were subjected to quantitative real time PCR utilizing TaqMan probes (9) to determine whether the immunoprecipitate was enriched for the p21$^{WAF1/CIP1}$ upstream chromatin sequences of interest. QRT-PCR was carried out using a Cepheid SmartCycler thermocycler, with the associated SmartCycler v.2.0 software used to analyze the data and determine the threshold count (C_t) for each reaction. The fluorophore used was Sybr Green, and the sequences of the primers and probes were the same as those used in the initial ChIP reaction described above. Real time PCR cycles started with an initial 1.5 minute denaturation step at 95° C, followed by 30 to 40 cycles of denaturation at 95° C for 10 seconds; annealing at 56° for 20 seconds; and extension at 72° C for 30 seconds. Each sample was run in triplicate and C_t determined for the target gene. To normalize the signal for each ChIP target and identify which targets were enriched in the chromatin by *Gax* expression, immunoprecipitate and flow-through target gene levels were normalized to β-actin sequence levels using the $\Delta\Delta C_t$ method (50), using described previously (24, 28), and the result presented as a ratio to chromatin-bound sequence/unbound. Differences in the target/β-actin ratio were evaluated using one-way ANOVA, followed by the Bonferonni post-test.

Electrophoretic mobility shift and supershift assays

To explore the possible direct binding sites of Gax on p21$^{WAF1/CIP1}$ genomic DNA, the p21$^{WAF1/CIP1}$ ChIP positive binding fragment A was analyzed by probes approximately 30 bp long as listed in Table 2. To label the probes, 100 ng purified PCR products, 5ul (25 μCi) γ-^{32}P-ATP, 1 μL of 10X T4 Kinase Buffer and 0.4 μL T4 Kinase, H_2O to a final volume of 10 μL were set up and incubated at 37° C 1 hr. G-50 Micro-columns (Amersham) were used to isolate

the labeled probes from unlabeled. Binding reactions of protein and DNA were then carried out in the following reaction mixture: 1x binding buffer (50 mM Tris pH 7.5, 25 mM NaCl, 3.5 μM $MgCl_2$,0.5 uM EDTA, 5% Glycerol,0.05% NP-40,and 0.25mg/ml BSA), 5 uM DTT, 50 ng/μL poly(dI-dC), 50,000 cpm labeled probe, 0, 100 ng, and 200 ng protein in 20 μL. Binding reactions were carried out at room temperature for 20 min. The non-denaturing 4% acrylamide gel was pre-run for 30 min at 4°C by at a voltage of 350 V, and then the protein-DNA binding complexes were load into the gel and run at 120 V at 4° C for 1-2 hrs. The gel was dried for 45 min and exposed to film at -80°C. For the supershift assay, the binding reaction was as follows: 1x binding buffer (50 μM Tris pH 7.5, 25 μM NaCl, 3.5 μM $MgCl_2$, 0.5 μM EDTA, 5% Glycerol, 0.05% NP-40,and 0.25 mg/ml BSA), 5 μM DTT, 50 ng/μL poly(dI-dC), 100-500 ng protein, 2 μL of anti-Flag in a total volume of 20 μL. Reaction is on ice for 20 min. Then 50,000 cpm of labeled probe (1-2 μL) was added mixed and put at room temperate for 10 min.

RESULTS

Gax induces G_0/G_1 cell cycle arrest in vascular ECs

Although we have previously shown that *Gax* inhibits EC proliferation (23), we wished to verify that the inhibition of EC proliferation due to *Gax* expression is because of G_0/G_1 cell cycle arrest (60). Consequently, we transduced HUVECs with either Ad.*Gax* or Ad.GFP for 12-16 hours, incubated them in low serum medium for 24 hours in order to induce quiescence, stimulated them with 10% serum, and harvested them an additional 24 hours after stimulation. We then stained them with propidium iodine and subjected them to flow cytometry. *Gax* significantly inhibited HUVEC reentry into the cell cycle, with a marked increase in the G_0/G_1 fraction and a concomitant decrease in the S-phase fraction (Figure 1A). In addition, Gax protein expression induced p21$^{WAF1/CIP1}$ expression in a dose-dependent manner and that overexpressing

Gax using adenoviral constructs did not induce p53 (Figure 1B), consistent with previous observations in vascular smooth muscle cells and fibroblasts, in which the induction of p21$^{WAF1/CIP1}$ by *Gax* was observed to be p53-independent (60).

Construction of Gax deletions

Next, in order to test which domains of the Gax protein are important for the ability of Gax to activate p21 expression, we made multiple *Gax* truncates, inserted them into the pCDNA3.1 expression vector (Invitrogen), and tagged them with Flag at the N-terminus. These constructs (Figure 2A) included: Flag-hugax (full length); Flag-hugaxNT (N-terminal domain lacking the homeodomain and C-terminal domain; Flag-hugaxΔCT (deletion of C-terminal domain; Flag-hugaxΔNT (deletion of N-terminal domain); Flag-hugaxΔHD (deletion of the homeodomain). These same deletion constructs were also inserted into the LRZSΔ vector for generation of retroviral expression vectors. To verify production and activity of the full Gax protein produced by the expression construct, we transduced HUVECs with pCDNA3.1 vectors expressing Flag-hugax for 24 hours as described in the Materials and Methods section and then harvested the cells to prepare extracts for Western blot using anti-Flag antibody. We observed expression of Gax protein and appropriate induction of p21$^{WAF1/CIP1}$ expression (Figure 2B). Protein expression for the remaining constructs was then verified by Western blog using anti-Flag antibodies (Figure 2C).

Deletion of the homeodomain abolishes nuclear localization of Gax.

Because Gax is a homeodomain protein and transcription factor, we wished to know what the effect of deleting these domains was on its nuclear localization. To investigate whether deletions of any of these domains affected the ability of the Gax protein to enter the nucleus, we transduced HUVECs with pCDNA3.1 constructs expressing the various deletions as described in Materials and Methods. After 24 hours, cells were harvested for nuclear and cytoplasmic

297 extracts. These extracts were subjected to Western blot with anti-Flag antibody, and we found
298 that the two constructs lacking a homeodomain were retained in the cytoplasm (Figure 3A),
299 whereas the other constructs localized to the nucleus. We confirmed this observation by
300 transducing HUVECs with the same deletion constructs, allowing the cells to incubate for 24
301 hours, and then fixing them and performing immunofluorescence using anti-Flag antibody
302 followed by confocal microscopy. Again, without its homeodomain, Gax remains mostly
303 localized in the cytoplasm (Figure 3B). Deleting either its N-terminal or C-terminal domains did
304 not affect nuclear localization of the *Gax* protein (Figure 3B).

305 ***Activation of p21WAF1/CIP1 expression by Gax requires the Gax homeodomain and the N-***
306 ***terminal domain***

307 Next, to determine which domains of the Gax protein are involved in activating
308 p21$^{WAF1/CIP1}$ expression, we cotransfected our *Gax* deletion constructs with a reporter construct
309 containing the p21$^{WAF1/CIP1}$ promoter upstream of Luciferase. We observed that, as expected,
310 deletion of the homeodomain completely abolished the ability of *Gax* to transactivate the
311 p21$^{WAF1/CIP1}$ promoter (Figure 3C). In contrast, deleting the C-terminal domain decreased, but
312 did not abolish, transactivation of the p21$^{WAF1/CIP1}$ promoter, but deleting the N-terminal domain
313 had a stronger effect, decreasing the ability of *Gax* to transactivate the p21$^{WAF1/CIP1}$ promoter
314 almost as much as did deleting the homeodomain (Figure 3C).

315 ***Deletion of the CAX repeat coding for a polyhistidine/glutamine region abolishes***
316 ***transactivation***

317 Having observed that deleting the N-terminal domain of the Gax protein abolished its
318 ability to transactivate the p21$^{WAF1/CIP1}$ promoter, we noted that, contained within the N-terminal
319 domain of *Gax*, is a $(CAX)_n$ repeated motif, also known as an *opa* or M repeat (65). This motif is
320 frequently found in developmentally regulated genes in *Drosophila*, particularly nuclear proteins
321 , and is also found in other homeobox genes, such as *HOXA1* (46), *Dfd* (53), and *Antp* (57). In

Gax, an *opa* repeat near the N-terminus of the protein codes for an 18 amino acid polyhistidine/glutamate tract consisting of twelve straight histidine residues followed by six residues consisting of four glutamates and two histidines (25). We wished to determine whether this domain functioned in the trans-activation of the p21$^{WAF1/CIP1}$ promoter (Figure 4A). To this end, we constructed another *Gax* deletion construct, this time lacking only the *opa* repeat and tested its ability to transactivate the p21$^{WAF1/CIP1}$ promoter by cotransfecting pCDNA3.1-hugaxΔCAX with the p21$^{WAF1/CIP1}$ reporter construct. We observed that deleting the CAX repeat completely abolished the ability of *Gax* to activate the p21$^{WAF1/CIP1}$ promoter (Figure 4B), indicating the importance of this motif for transcriptional activation by *Gax*. Indeed deleting the CAX repeat appears to result in *Gax* producing mild repression of the p21$^{WAF1/CIP1}$ promoter.

Gax binds to sequence 15 kb upstream of the p21$^{WAF1/CIP1}$ start codon through its homeodomain

Although we have reported that *Gax* transactivates the p21$^{WAF1/CIP1}$ promoter (60), it is as yet unknown to what sequence the Gax protein binds to activate transcription. We therefore asked whether Gax binds in living cells to specific sequences upstream of p21$^{WAF1/CIP1}$. Because we did not know *a priori* where Gax binds in the p21 promoter, we performed chromatin immunoprecipitation (ChIP) using primers designed to sample the chromatin at regular intervals using 200 bp amplicons, beginning at the start codon and proceeding to approximately 15 kb upstream (Figure 5A). HUVECs were transfected with Flag-hugax or hugax without Flag, incubated for 24 hours, and then harvested for ChIP using anti-Flag antibody. Surprisingly, the sequence to which Gax could bind that we identified first was located approximately 15 kb upstream of the p21$^{WAF1/CIP1}$ start codon (Sequence A, Figure 5B). Moreover, we measured the level of this sequence and compared it to all the other sequences used for ChIP by quantitative real time PCR using the same primers. We normalized the amplified p21$^{WAF1/CIP1}$ promoter

346 sequences to the signal obtained using a primer/probe set designed to detect and measure β-actin
347 sequence. These results indicated that Sequence A was greatly enriched by ChIP only in the anti-
348 Flag immunoprecipitates from cells that had been transduced with Flag-tagged *Gax*. None of the
349 other sequences was enriched in the chromatin, nor was Sequence A detected above any of the
350 other sequences when ChIP was carried out with vectors expressing *Gax* without the Flag tag
351 (Figure 5C). These results confirm that there is an *in vivo* binding site for Gax approximately 15
352 kb upstream from the transcriptional start site of the p21$^{WAF1/CIP1}$ gene.

353 Given these findings, we next wished to verify that Gax binds this upstream chromatin
354 sequence through its DNA-binding homeodomain. Consequently, we repeated the ChIP assay;
355 only this time we used the Flag-tagged *Gax* deletion constructs that we had made initially
356 (Figure 2A) and then performed quantitative real time PCR as before (Figure 5C), to see which
357 of these constructs produced an immunoprecipitate enriched in Sequence A. We found that none
358 of the constructs that lacked the *Gax* homeodomain (hugax-NT, hugaxΔHD) resulted in
359 enrichment for Sequence A, whereas constructs containing the *Gax* homeodomain (wild-type
360 hugax, hugaxΔNT, and hugaxΔCT) all did (Figure 5D). These results indicate that Gax binds to
361 this upstream chromatin sequence (Sequence A) through its homeodomain.

362 ***Identification of an ATTA-containing core binding site for Gax***

363 Because Sequence A is 200 bp long, we wished to identify where in Sequence A Gax
364 binds. To this end, we designed several overlapping probes for use in electrophoretic mobility
365 shift assays (Table 2 and Figure 6A), to determine which sequence was bound by Gax. We found
366 that A6 strongly bound Gax in EMSAs using recombinant Gax protein (Figure 6B), and
367 supershifts using a previously described anti-Gax antibody (59) demonstrated that the Gax
368 protein was bound in this complex (Figure 6C).

The sequence of A6 contained an AT-rich sequence, with ATTACAATTA at its core. Because this resembles the DNA binding sites of other homeodomain proteins, we systematically mutated residues beginning one residue to the 5' and 3' end of this core sequence (labeled A6Mt1 through A6Mt11) and repeated the EMSAs. Mutating residues in the second ATTA decreased Gax binding markedly, and, indeed, in particular, mutating the first T (A6Mt9) abolished binding completely (Figure 6D).

Lastly, to study the functional consequences of altering the sequence of this AT-rich site, we constructed three expression vectors (Figure 7A). One (p21A6-Luc) contained the A6 sequence inserted upstream of a minimal promoter and a Luciferase reporter. The others contained A6Mt1 upstream of Luciferase (p21A6Mt1-Luc), because this mutation resulted in stronger Gax binding, and A6Mt11 (p21A6Mt11-Luc), because this mutant completely abolished Gax binding (Figure 6C). We then cotransfected pCDNA3.1-hugax with the reporters containing these mutated A6 sequences and observed that *Gax* effectively trans-activated the reporter containing sequence A (p21A-Luc). In contrast, neither mutant (p21A6Mt1-Luc or p21A6Mt11-Luc) could be trans-activated by *Gax* (Figure 7B). From this, we conclude that the AT-rich site that we have identified is an important regulatory element contributing to the activation of p21$^{WAF1/CIP1}$ expression by *Gax*.

Finally, we asked whether other AT-rich sequences resembling the AT-rich sequence in A6 could also be bound and transactivated by *Gax*. We searched the entire 15 kb sequence that we had surveyed with ChIP and located four additional such sequences, two in Fragment B, one in Fragment C, and one in Fragment F. All were able to bind Gax *in vitro* in electrophoretic mobility shift assays (Figure 8A). Because we had observed regulation of p21$^{WAF1/CIP1}$ expression before, we were most interested in determining whether the sequence on fragment C

contributed to regulation of p21$^{WAF1/CIP1}$ promoter activity. Consequently, we deleted the two ATTA regions in this fragment C, inserted it upstream from Luciferase as above, and then performed cotransfection experiments. With increasing ratios of pCDNA3.1-hugax, we noted increasing Luciferase activity from the plasmid in which fragment C was driving Luciferase (p21C-Luciferase). However, deleting the ATTA sequences in fragment C (p21C-mut-Luciferase) nearly completely abolished Luciferase activity compared to fragment C (Figure 8B). These results suggest that this AT-rich site is active as well in regulating Gax expression.

DISCUSSION

Interactions between tumors and their surrounding stroma are critical in regulating the growth and metastasis of tumors. One particularly important interaction is the induction of angiogenesis by tumors. Critical to the early phenotypic changes that ECs undergo during angiogenesis is reentry into the cell cycle. Thus, determining the factors that regulate EC proliferation is critical to understanding the process of angiogenesis and developing therapeutic strategies to block it. One such strategy is to target EC proliferation in response to proangiogenic factors, such as VEGF (19) or bFGF (63), and strategies targeting VEGF have proven successful in the treatment of colorectal and breast cancer (31, 42). Another strategy to target EC proliferation as part of antiangiogenic therapy is to target signaling pathways downstream from proangiogenic factors and the transcriptional programs that they activate. Towards this end, we have been studying the homeobox gene *Gax*, because it has many characteristics that suggest it as an important negative regulator of EC proliferation. Given its activity in VSMCs (25, 48, 60, 64, 66) and more recent results showing that *Gax* activates p21$^{WAF1/CIP1}$ in ECs (23, 47), we wished to study in more detail how *Gax* induces p21$^{WAF1/CIP1}$ expression. Specifically, we were interested in (1) determining which domains of the Gax protein might be responsible for this

activity of *Gax* and (2) identifying potential DNA binding sites responsible for activation of the p21$^{WAF1/CIP1}$ promoter. To this end, we used two strategies. First, we constructed a series of deletions of the *Gax* cDNA and determined which domains are important for activation of p21$^{WAF1/CIP1}$ expression. Our results indicate that the homeodomain is critical for this function. Moreover, the CAX (*opa*) (65) repeat is also clearly involved in transcriptional activation. We note that the expansion of the CAX repeats of other homeodomain genes have been associated with neurodegenerative diseases and autism (46).

Next, we used ChIP to identify putative *Gax* binding sites in the chromatin upstream from the p21$^{WAF1/CIP1}$ transcriptional start site. The results of this assay identified an ATTA-containing sequence to which Gax bound strongly. We note that this sequence strongly resembles a universal homeobox DNA consensus sequence (32, 33). (See Figure 8) and that the results of our experiments in which we mutated individual residues within this binding site agree with results showing the importance of a CAATTA core sequence in the upstream (33), suggesting that this is one functional DNA sequence to which the Gax protein can bind and activate transcription. Also, we have shown that, not only is this site bound by Gax *in vivo* in the ChIP assay, but it is also able to activate transcription of a minimal promoter as demonstrated by cotransfection experiments, an activity that is abolished by mutating individual residues (Figure 7). Finally, based on the discovery of this site 15 kb upstream of the translational start site of p21$^{WAF1/CIP1}$, we noted that another AT-rich site in the core p21$^{WAF1/CIP1}$ promoter is also bound by Gax and able to activate transcription in cotransfection assays (Figure 8), for at least two likely sites to which Gax can bind and thereby induce p21$^{WAF1/CIP1}$ expression.

The importance of homeobox genes in the regulation of endothelial cell phenotype during tumor-induced angiogenesis is becoming increasingly more apparent, with the recent

descriptions of homeobox genes that promote (6-8, 13, 16, 39, 43, 67) or block (23, 44, 45, 47, 54) the angiogenic phenotype. We have now shown that the homeobox gene *Gax*, which has previously been shown to inhibit angiogenesis in the peripheral vasculature (23, 47), upregulates p21WAF1/CIP1 expression through a direct interaction with an enhancer 15 kb upstream from the transcriptional start site, and we have identified one putative *Gax* binding site. Moreover, we have shown that it is the *Gax* homeodomain that is responsible for binding to this site and that the polyhistidine (*opa*) repeat is involved in mediating transcriptional activation of the p21$^{WAF1/CIP1}$ gene. These data suggest that *Gax* is an important regulator of EC proliferation and angiogenesis. Moreover, given how *Gax* inhibits NF-κB signaling, the identification of a putative *Gax* binding site responsible for the activation of p21$^{WAF1/CIP1}$ expression will permit us to narrow down candidate chromatin sequences in NF-κB-dependent genes that might mediate the ability of *Gax* to block activation of target genes by NF-κB, as well as other *Gax*-dependent promoters. These studies will allow us to determine the molecular mechanisms by which *Gax* inhibits EC activation and angiogenesis, as well as suggesting potential strategies for inhibiting angiogenesis by modulating *Gax* activity.

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FIGURE LEGENDS

Figure 1. ***Gax expression causes G_0/G_1 cell cycle arrest in HUVECs.*** **A. Gax induces cell cycle arrest.** HUVECs were incubated in low serum (0.1%) medium for 24 hrs, after which they were stimulated with 10% serum and 5 ng/ml VEGF for 24 hours and then harvested for flow cytometry. There was a marked increase in the G_0/G_1 fraction ($p < 0.05$) and a concomitant decrease in the S-phase fraction. B. Gax does not affect p53 levels in HUVECs. Subconfluent HUVECs were transduced with varying MOIs of Ad.Gax and then incubated overnight, after which they were harvested for protein extract, which was subjected to Western blotting for Gax and p53. Control cells were transduced with Ad.GFP at the same MOIs.

Figure 2. ***Construction of Gax deletions.*** **A. Strategy.** Gax deletions were constructed as described in the Materials and Methods section according to the strategy outlined in this figure. Constructs were made in which either the homedomain, N-terminal domain, or C-terminal domains were deleted. See text for more details. **B. Protein expression and function by full length Gax construct.** HUVECs were transfected with the the pCDNA3.1 construct containing the full-length human Gax cDNA tagged with Flag and then harvested for Western blot 24 hours later. Western blots were carried out using anti-Flag and anti-p21$^{WAF1/CIP1}$ antibodies. Strong Gax expression was observed, and p21$^{WAF1/CIP1}$ was induced as previously observed with the adenoviral constructs (23, 60). **C. Protein expression by Gax deletion constructs.** HUVECs were transfected with the completed pCDNA3.1 and LZRSΔ Gax deletion constructs as in **B** and then incubated for 24 hours in standard medium, after which they were harvested for protein for Western blot. Western blot was carried out with anti-Flag antibody. (**Legend:** 1. Empty vector

674 control; 2. Flag-hugax; 3. Flag-hugaxNT; 4. Flag-hugaxΔCT; 5. Flag-hugaxΔNT; 6. Flag-
675 hugaxΔHD.)

676 **Figure 3.** ***Effect of deleting different domains of the Gax protein on nuclear***
677 ***localization and transactivation of the p21WAF1/CIP1 promoter.*** **A. Effect on nuclear**
678 **localization.** HUVECs were transfected with the various pCDNA3.1 Gax constructs, incubated
679 for 24 hours, and then harvested for isolation of cytoplasmic and nuclear fractions as described in
680 the Materials and Methods section. The cytoplasmic fraction was subjected to Western blot with
681 anti-Flag antibodies, and it was noted that deleting the homeodomain greatly increased the level
682 of Gax in the cytoplasmic fraction. (**Legend:** 1. Empty vector control; 2. Flag-hugax; 3. Flag-
683 hugaxNT; 4. Flag-hugaxΔCT; 5. Flag-hugaxΔNT; 6. Flag-hugaxΔHD.) **B. Confocal**
684 **microscopy.** To verify the observation in A, we transfected HUVECs with the pCDNA3.1 Gax
685 constructs as before and incubated the cells for 24 hours. They were then subjected to
686 immunofluorescence with anti-Flag antibody as described in Materials and Methods, followed by
687 confocal microscopy. All constructs lacking the homeodomain failed to localize in the nucleus
688 and stayed in the cytoplasm. C. The effect of Gax deletions on transactivation of the p21$^{WAF1/CIP1}$
689 promoter. The various pCDNA3.1 Gax constructs were cotransfected with a p21$^{WAF1/CIP1}$
690 promoter-Luciferase construct previously used to demonstrate that *Gax* transactivates the
691 p21$^{WAF1/CIP1}$ promoter. Deleting the homeodomain abolishes transactivation. Similarly, deleting
692 the N-terminal domain dramatically decreases transactivation, whereas deleting the C-terminal
693 domain has less of an effect.

694 **Figure 4.** ***Function of the CAX/polyhistidine (opa) repeat.*** A. Deletion of the
695 CAX/polyhistidine repeat. B. Effect of deleting the CAX/polyhistidine repeat on the ability of
696 Gax to transactivate the p21$^{WAF1/CIP1}$ promoter. We compared the ability of full length Gax and

GaxΔCAX constructs to transactivate the p21WAF1/CIP1 promoter. Deleting the CAX repeat dramatically decreased transactivation and indeed may have produced mild repression.

Figure 5. ***Chromatin immunoprecipitation.*** A. Design of initial primer sets for chromatin immunoprecipitation assay. **B. Chromatin immunoprecipitation with the initial set of nine primer pairs.** HUVECs were transduced with pCDNA3.1 constructs expressing either hugax or Flag-hugax and then subjected to ChIP assay as described in Materials and Methods. Only primer pair A amplified a fragment that had been bound to chromatin, Fragment A. **C. Quantitative real time PCR.** The immunoprecipitates from the ChIP assay were subjected to quantitative real time PCR with the primer pairs used and normalized to β-actin. Only Fragment A was amplified at a ratio of greater than one compared to β-actin ($p<0.05$, two-way ANOVA). **D. Chromatin immunoprecipitation with Gax deletion constructs.** ChIP assays were carried out with HUVECs transduced with Gax deletion constructs using primer pair A. Enrichment of chromatin for Fragment A was only observed with *Gax* deletions containing the Gax homeodomain.

Figure 6. ***Identification of the 15 kb upstream binding site for Gax.*** **A. Probes for electrophoretic mobility shift assay.** Overlapping probes were designed to span the entire length (200 bp) of Fragment A as shown. **B. Electorphoretic mobility shift assays.** The various probes from **A** were end-labeled with γ-^{32}P-dATP and then subjected to EMSA using nuclear extracts from HUVECs transduced with full length Gax expression construct. Only Fragment A6 produced a band shift. **C. Supershift of binding activity to Fragment A6 using anti-Gax antibody.** The nuclear extracts in B were subjected to supershifts using fragment A6 and anti-Gax antibody (59). A marked supershift was observed. **D. Site-directed mutagenesis of the AT-rich core of Fragment A6.** Individual nucleotides in the ATTACAATTA core of the putative

Gax binding site in Fragment A6 were mutated, end-labeled, and subjected to EMSA, as described in Materials and Methods. Mt8 strongly reduced Gax binding to its target sequence, and Mt9 and Mt11 abolished Gax binding.

Figure 7. ***Effect of mutating the 15 kb upstream binding site for Gax on transactivation by Fragment A.*** **A. Promoter constructs.** The A6 fragments subjected to site-directed mutagenesis were inserted upstream of Luciferase in a promoter/reporter construct as described in Materials and Methods. **B. Effect of mutations in A6 on transactivation by Gax.** The constructs described in A were cotransfected with pCDNA3.1-Flag-hugax at different ratios. Both the A6Mt1 and A6Mt11 abolished transactivation, even though A6Mt1 did not abolish binding, as shown in Figure 6D.

Figure 8. ***Identification of an additional AT-rich sequence to which Gax can bind and activate transcription.*** **A. Additional AT-rich sequences in the upstream chromatin of p21$^{WAF1/CIP1}$.** Additional AT-rich sequences containing ATTA repeats resembling the one identified by ChIP were located on the upstream chromatin of p21WAF1/CIP1. EMSAs were then performed using probes containing these sequences, as well as the sequence identified by ChIP (sequence A6) as a Gax binding site. **B. Effect deleting the ATTA repeat in the core p21WAF1/CIP1 promoter.** The AT-rich sequence containing the ATTA was deleted from sequence C and then the wild type (p21C) and mutated sequences (p21C-mut) extended to 1.5 kb using PCR and placed upstream of Luciferase in the pGL3 plasmid to produce p21C-Luciferase and p21C-mut-Luciferase. These constructs were then cotransfected with pCDNA3.1-Flag-hugax at different ratios as described in Materials and Methods. Deleting the sequence containing two ATTA elements nearly completely abolished transactivation by Gax.

TABLE 1. Primers for the initial ChIP assay

Region		Primer sequence (5' to 3')
A	Forward	CCC AGC AGA TAC AGG GTT GT
	Reverse	CTT GTC CTT GCC TTT GCT TC
B	Forward	GGT TAT CCT GCG TGT GAC CT
	Reverse	TTT GTA GTT GCC TCC CCT TG
C	Forward	CTT CAA GGC AGT GGG AGA AG
	Reverse	GAT TGT GGC TAA ACC CCA GA
D	Forward	CTC TCC AAT TCC CTC CTT CC
	Reverse	AGA AGC ACC TGG AGC ACC TA
E	Forward	TTC CCT CTC CGA AAG CTA CA
	Reverse	CAG CTC CAA GAT GCT TTT CC
F	Forward	AGC TTT CAC CCC CAG AAA CT
	Reverse	CCC TTC AGG AGA GGG AAA AC
G	Forward	CAC CTT TCA CCA TTC CCC TA
	Reverse	GCA GCC CAA GGA CAA AAT AG
H	Forward	ACC CCA GGT AAA CCT TAG CC
	Reverse	AGT TTG CAA CCA TGC ACT TG
I	Forward	GGT CAG GGG TGT GAG GTA GA
	Reverse	TGT GGC TCC AAA ATG ACA AA

TABLE 2: Probe sequences for identification of the Gax binding site within Sequence A

Sequence Name	Sequence
A1	CAA TGA TTC CTC CCA GCA GAT ACA GGG TT
A2	GTT AGA AAC CAC TGA GGA TAG GGA AGA GGG
A3	AGT GAC ATC TCC TCC CTG GCT CTG AAC TTG
A4	GCT CTA GTG TGG CCA CTA GCA TTT GGG GCT T
A5	GGG GGT TGC AGC TGT CGC ATT CCA CAG TGG
A6	CCC CGA TGG CAT TAC AAT TAC AGA TGA CAC T
A7	TAG AGC CAC CCT AGG GAA GCA AAG GCA AG
A7	AGA TAC AGG GTT GTT AGA AAC CA
A8	TAG GGA AGA GGG AGT GAC ATC TC
A9	GGC TCT GAA CTT GGC TCT AGT GTG GC
A10	CAT TTG GGG CTT GGG GGT TGC AGC TGT
A11	CCA CAG TGG CCC CGA TGG CAT T
A12	TAC AGA TGA CAC TTA GAG CCA CCC TA

Figure 1

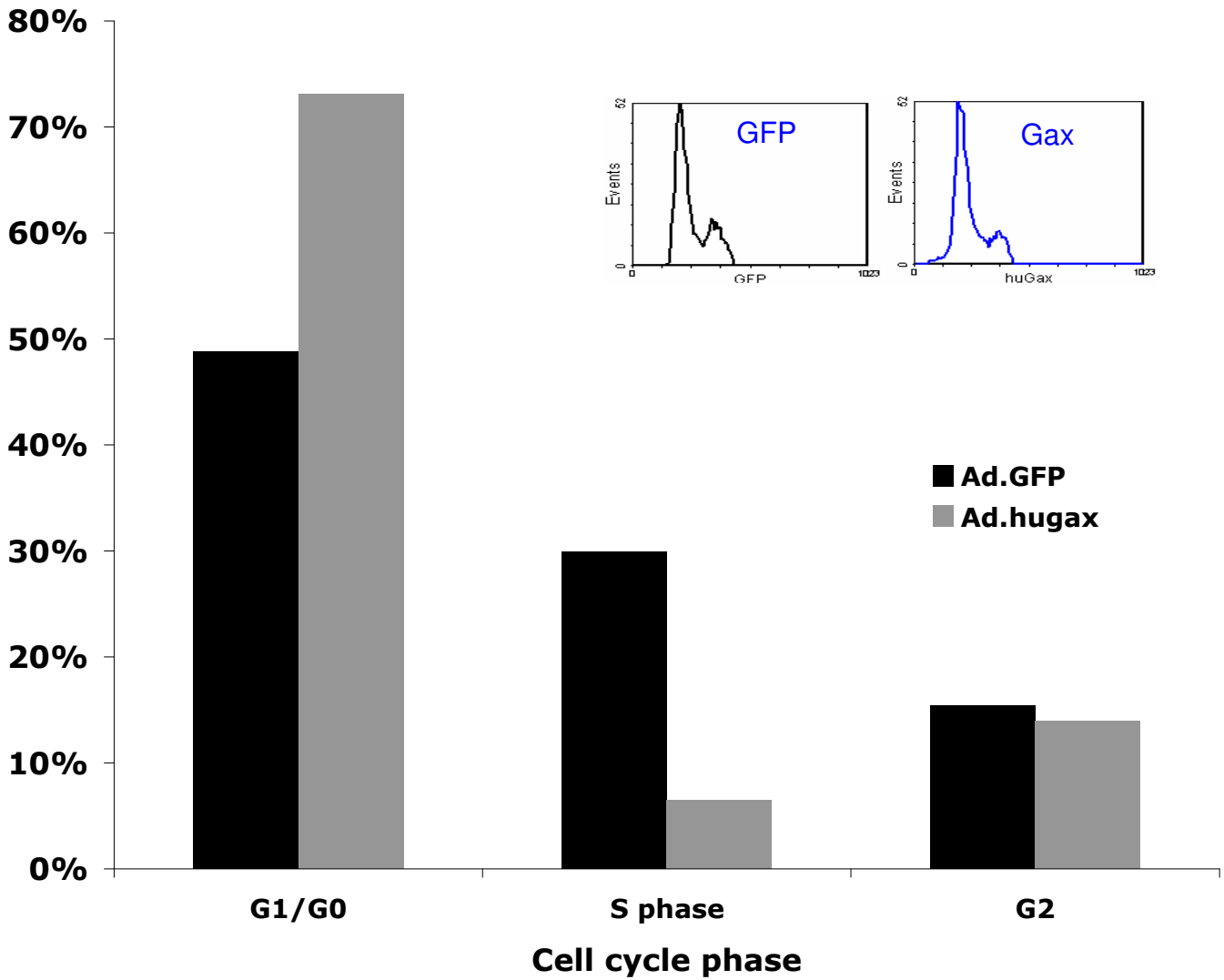

80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP
Gax
Events
GFP
huGax
Ad.GFP
Ad.hugax
G1/G0
S phase
G2
Cell cycle phase

Figure 1B

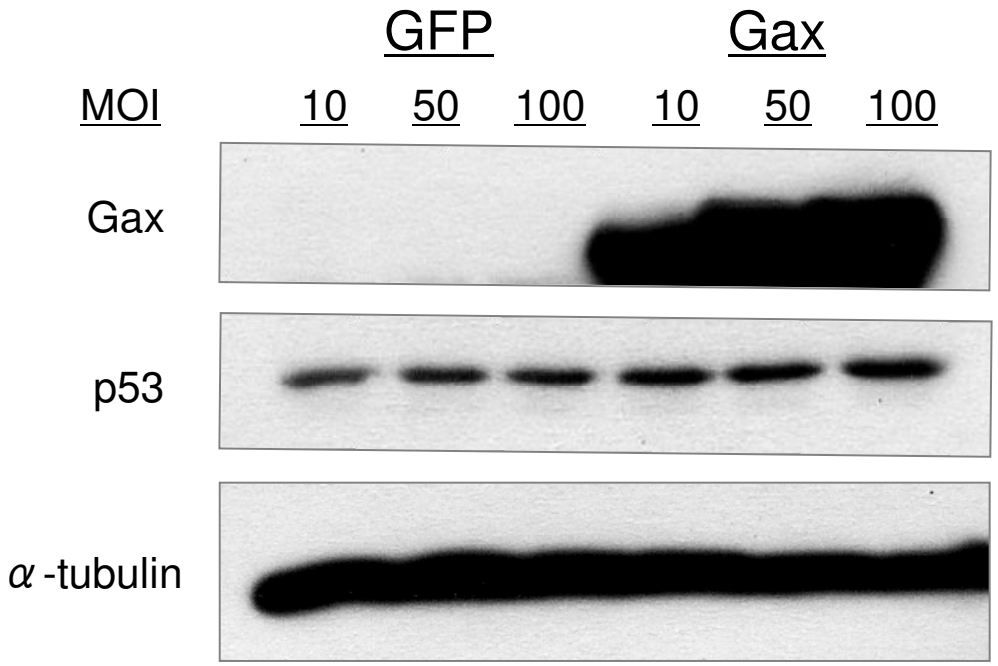
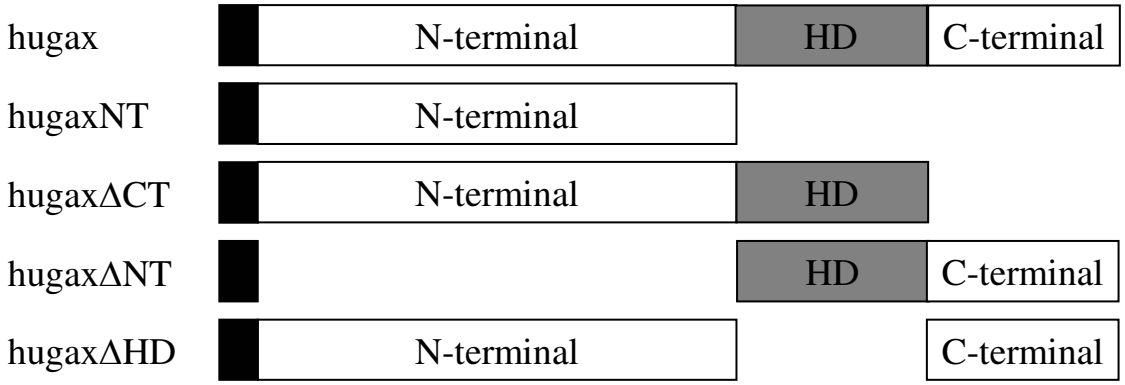


Figure 2A


hugax
N-terminal
HD
C-terminal
hugaxNT
N-terminal
hugaxΔCT
N-terminal
HD
hugaxΔNT
HD
C-terminal
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N-terminal
C-terminal

Figure 2B

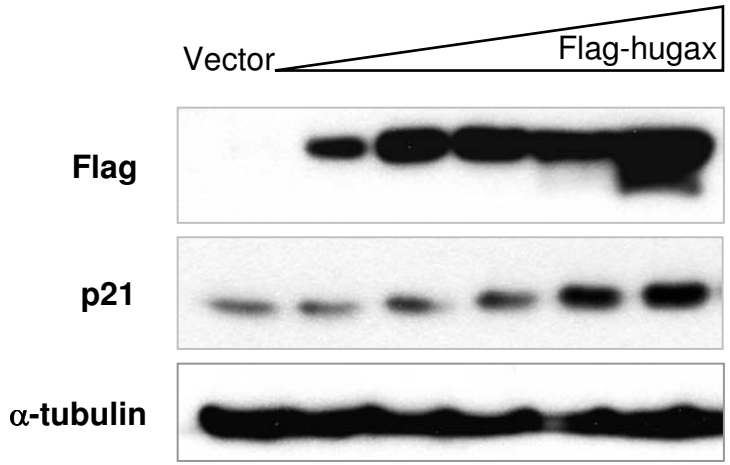


Figure 2C

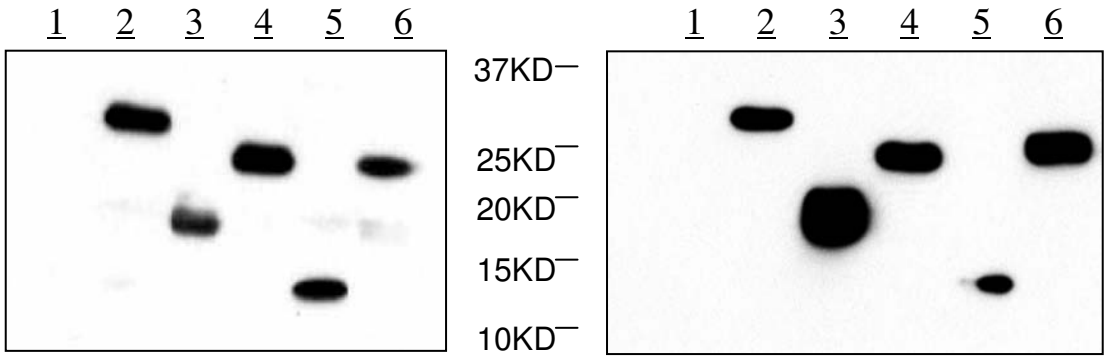


Figure 3A

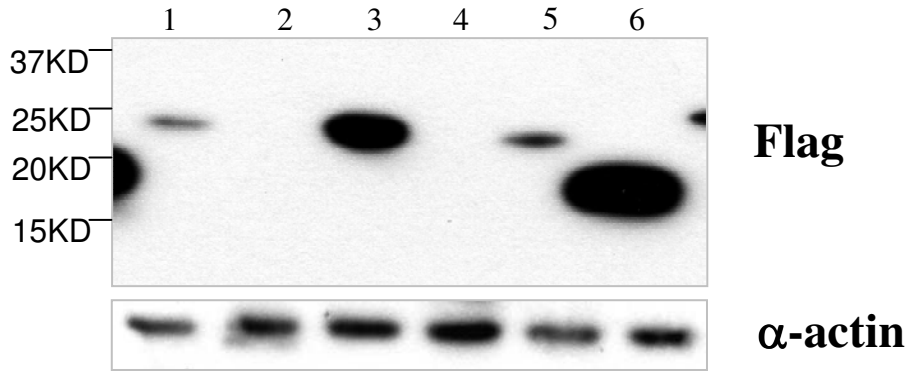


Figure 3B

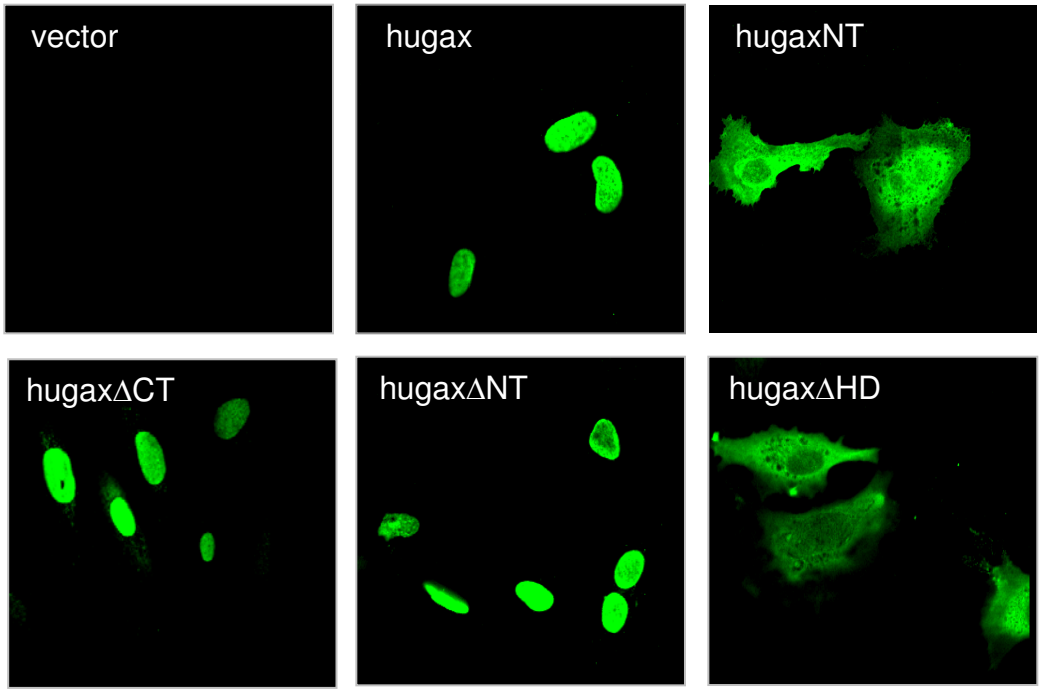


Figure 3C

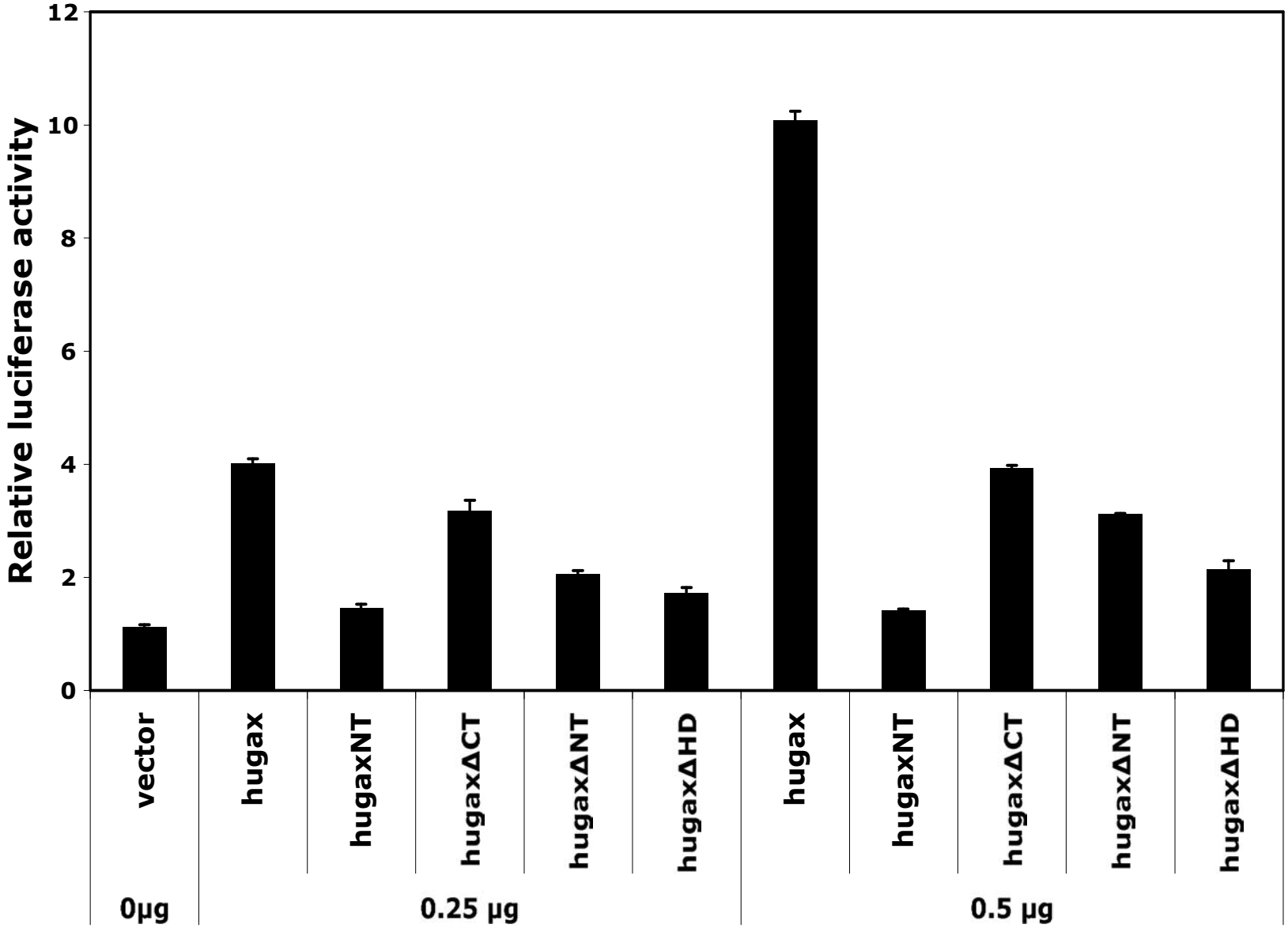

Relative luciferase activity
12
10
8
6
4
2
0
vector
hugax
hugaxNT
hugaxΔCT
hugaxΔNT
hugaxΔHD
hugax
hugaxNT
hugaxΔCT
hugaxΔNT
hugaxΔHD
0μg
0.25 μg
0.5 μg

Figure 4A

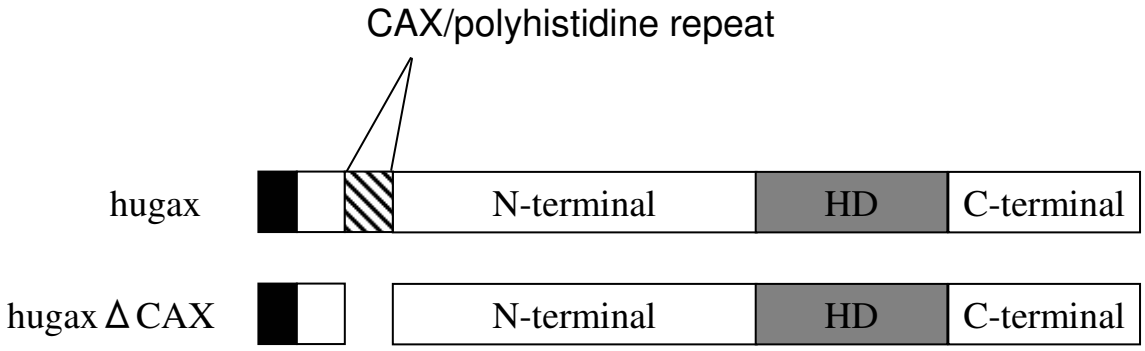

CAX/polyhistidine repeat
hugax
N-terminal
HD
C-terminal
hugax ΔCAX
N-terminal
HD
C-terminal

Figure 4B

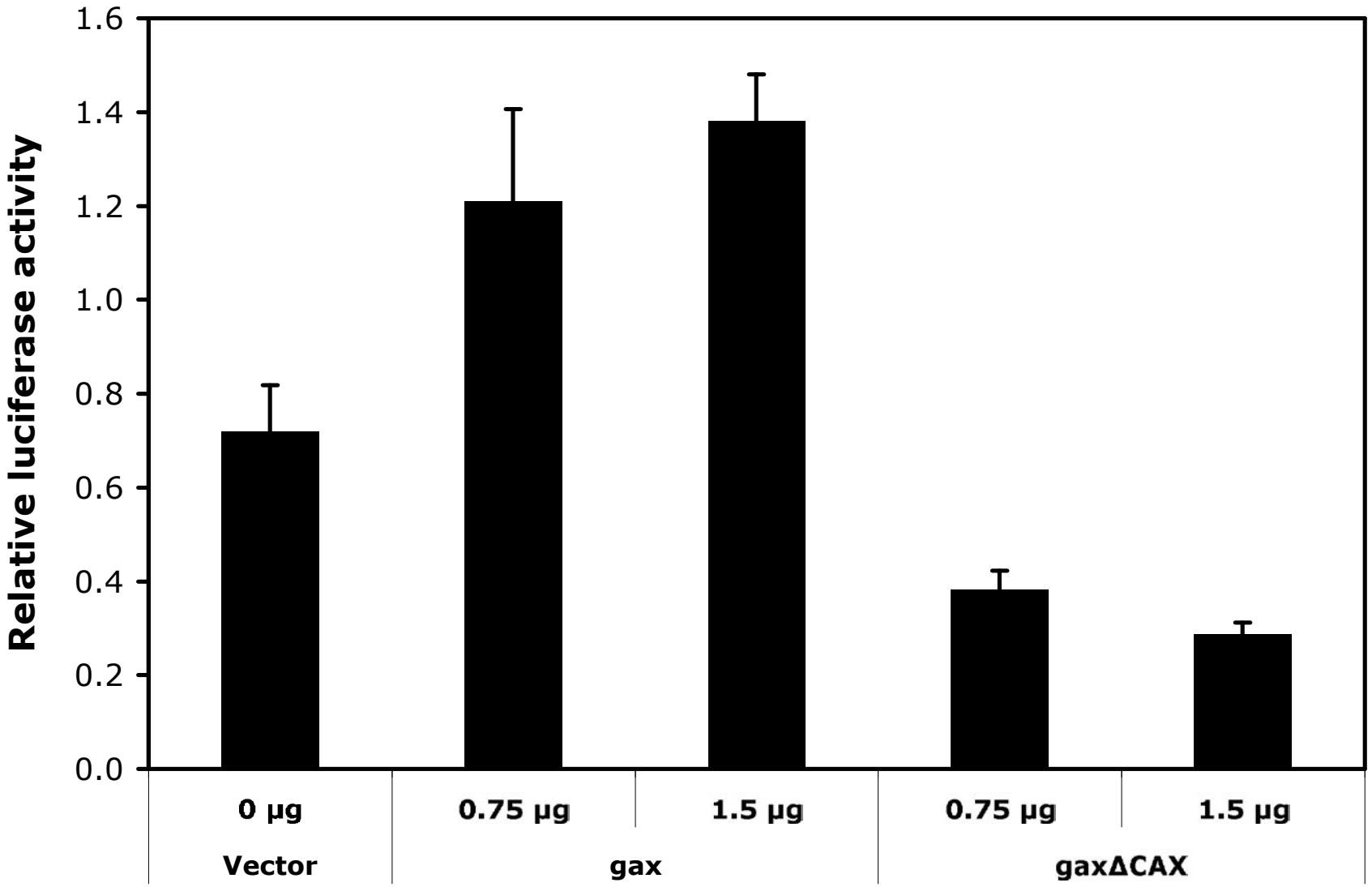

Relative luciferase activity
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 µg
0.75 µg
1.5 µg
0.75 µg
1.5 µg
Vector
gax
gaxΔCAX

Figure 5A

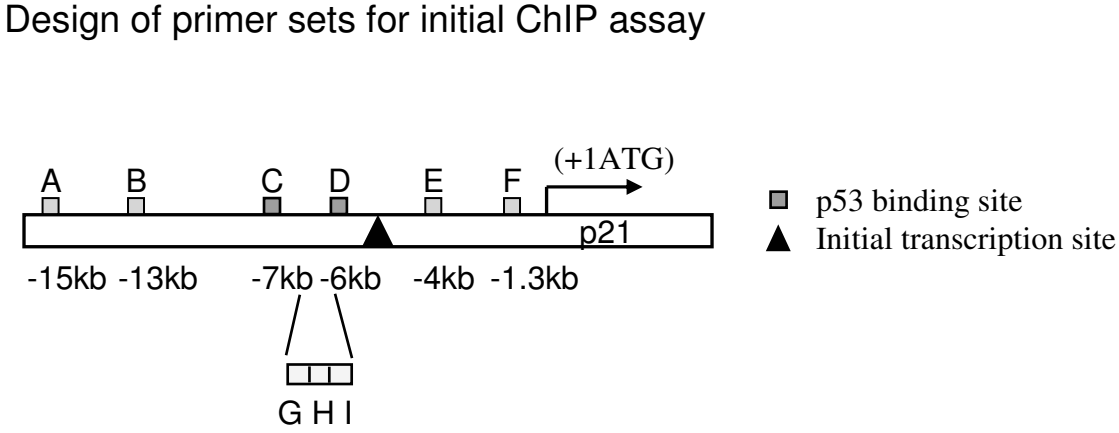

Design of primer sets for initial ChIP assay
A
B
C
D
E
F
(+1ATG)
p21
-15kb
-13kb
-7kb
-6kb
-4kb
-1.3kb
G H I
p53 binding site
Initial transcription site

Figure 5B

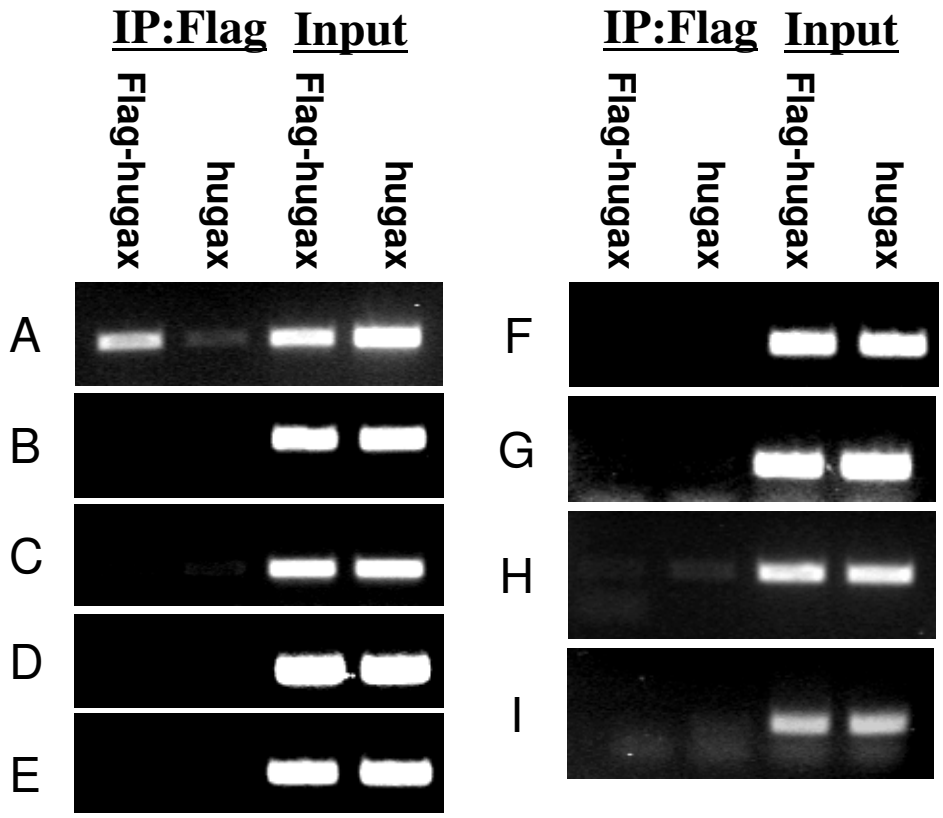

IP:Flag
Input
IP:Flag
Input
Flag-hugax
hugax
Flag-hugax
hugax
Flag-hugax
hugax
Flag-hugax
hugax
A
B
C
D
E
F
G
H
I

Figure 5C

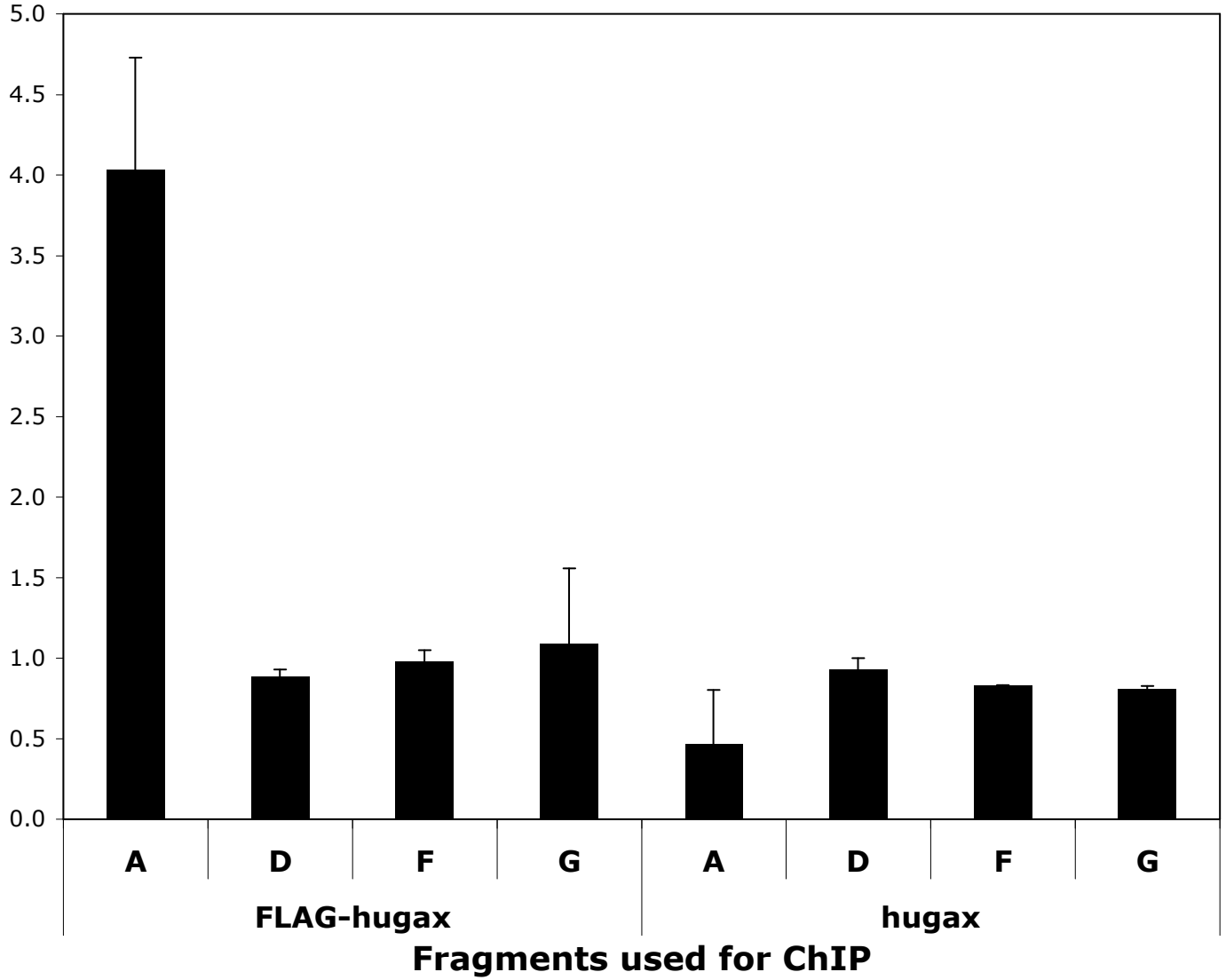

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
D
F
G
A
D
F
G
FLAG-hugax
hugax
Fragments used for ChIP

Figure 5D

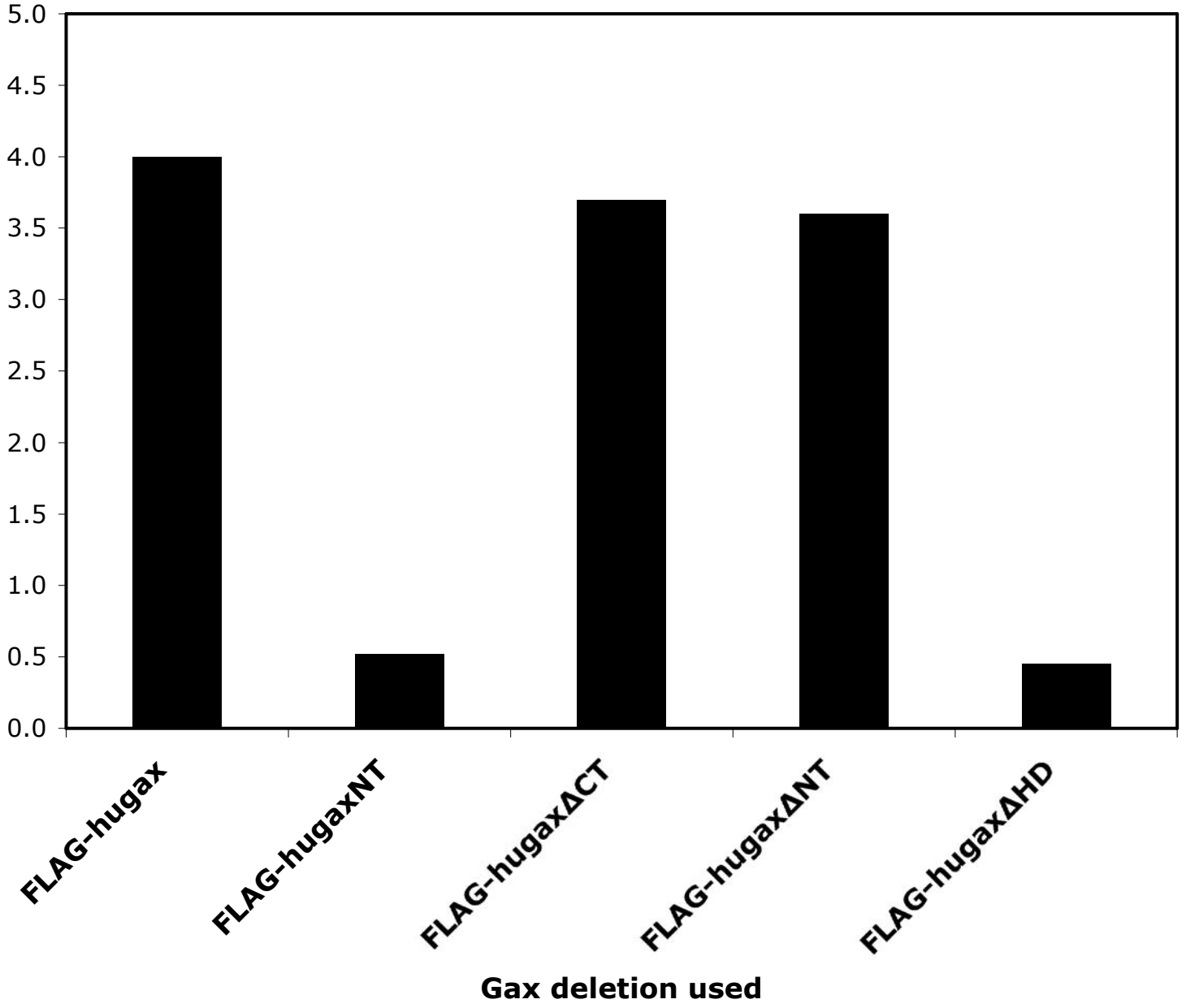

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
FLAG-hugax
FLAG-hugaxNT
FLAG-hugaxΔCT
FLAG-hugaxΔNT
FLAG-hugaxΔHD
Gax deletion used

Figure 6A

p21 fragment A:
CAATGATTCCTCCCAGCAGATACAGGGTTGTTAGAAACCACTGAGGATAGGGAAGAGGGAGTGACATCTCC
TCCCTGGCTCTGAACTTGGCTCTAGTGTGGCCACTAGCATTTGGGGCTTGGGGGTTGCAGCTGTCGCATTC
CACAGTGGCCCCGATGGCATTACAATTACAGATGACACT

p21 fragment A1:CAATGATTCCTCCCAGCAGATACAGGGTT
p21 fragment A2:GTTAGAAACCACTGAGGATAGGGAAGAGGG
p21 fragment A3:AGTGACATCTCCTCCCTGGCTCTGAACTTG
p21 fragment A4:GCTCTAGTGTGGCCACTAGCATTTGGGGCTT
p21 fragment A5:GGGGGTTGCAGCTGTCGCATTCCACAGTGG
p21 fragment A6:CCCCGATGGCATTACAATTACAGATGACACT
p21 fragment A7:AGATACAGGGTTGTTAGAAACCA
p21 fragment A8:TAGGGAAGAGGGAGTGACATCTC
p21 fragment A9:GGCTCTGAACTTGGCTCTAGTGTGGC
p21 fragment A10:CATTTGGGGCTTGGGGGTTGCAGCTGT
p21 fragment A11:CCACAGTGGCCCCGATGGCATT

Figure 6B

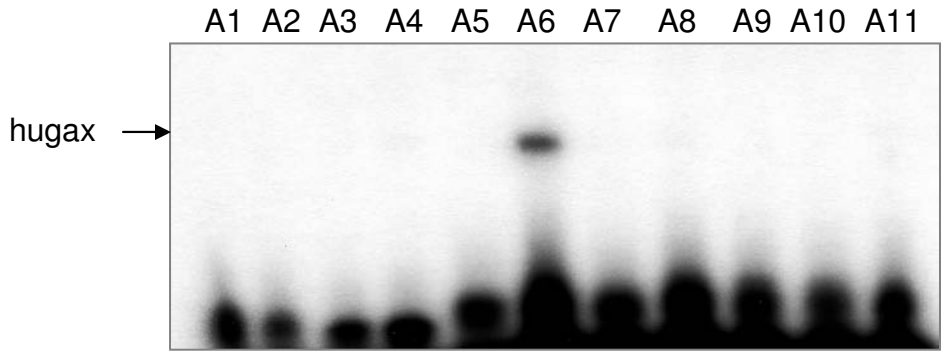


Figure 6C

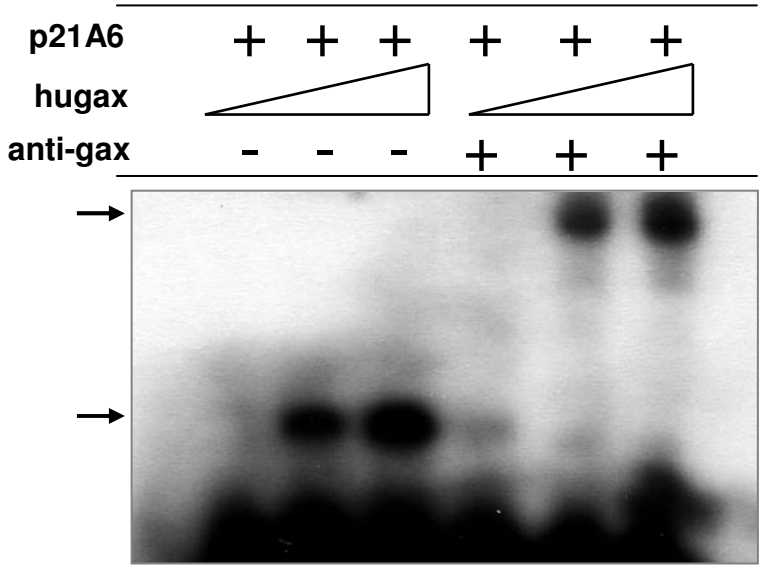


Figure 6D

```
p21A6:      CCCCGATGGCATTACAATTACAGATGACACT
Mt1         CCCCGATGGAATTACAATTACAGATGACACT
Mt2         CCCCGATGGCGTTACAATTACAGATGACACT
Mt3         CCCCGATGGCAGTACAATTACAGATGACACT
Mt4         CCCCGATGGCATGACAATTACAGATGACACT
Mt5         CCCCGATGGCATTGCAATTACAGATGACACT
Mt6         CCCCGATGGCATTAAAATTACAGATGACACT
Mt7         CCCCGATGGCATTACGATTACAGATGACACT
Mt8         CCCCGATGGCATTACAGTTACAGATGACACT
Mt9         CCCCGATGGCATTACAAGTACAGATGACACT
Mt10        CCCCGATGGCATTACAATGACAGATGACACT
Mt11        CCCCGATGGCAGTACAAGTACAGATGACACT

****
```

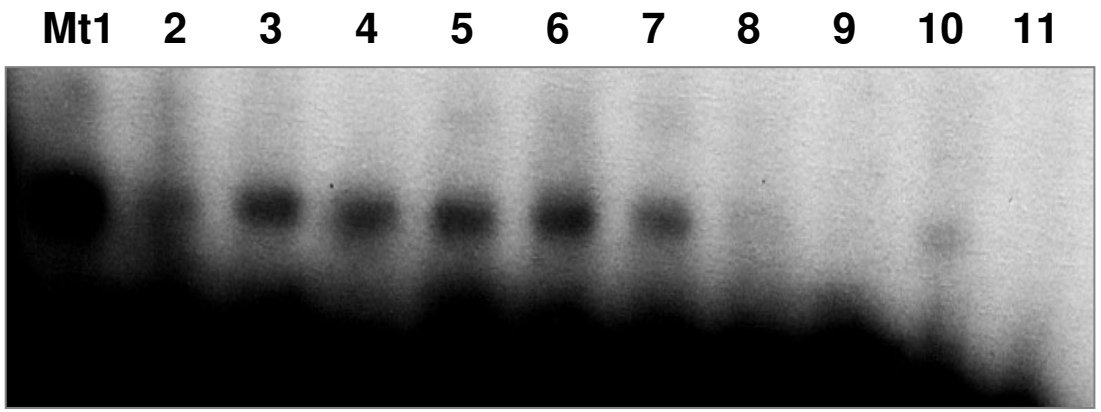


Figure 7A


p21A6-luc
CCCCGATGGCATTACAATTACAGATGACACT
luc
p21A6Mt1-luc
CCCCGATGGAATTACAATTACAGATGACACT
luc
p21A6Mt11-luc
CCCCGATGGCAGTACAAGTACAGATGACACT
luc

Figure 7B

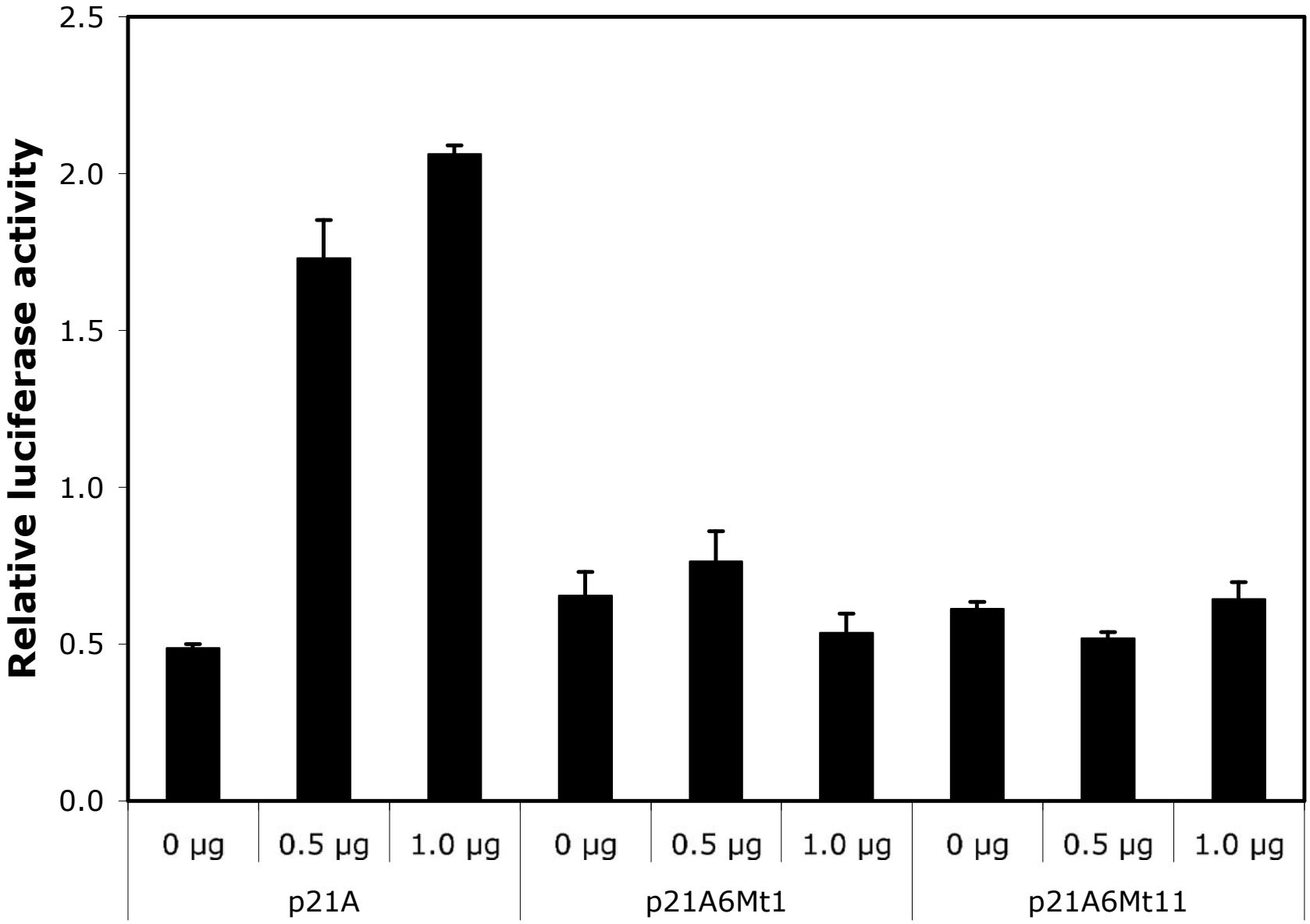

Relative luciferase activity
2.5
2.0
1.5
1.0
0.5
0.0
0 µg
0.5 µg
1.0 µg
0 µg
0.5 µg
1.0 µg
0 µg
0.5 µg
1.0 µg
p21A
p21A6Mt1
p21A6Mt11

Figure 8A

Potential gax binding sites in upstream p21$^{WAF1/CIP1}$ chromatin

```
Fragment A:   GTGGCCCCGATGGCATTACAATTACAGATGACA
Fragment B1:  TCTGTACTAAAAACTATTAAAAAATTAGCCAGG
Fragment B2:  AATCTCAGTTTGCCCATTAATATTATAGGTCTG
Fragment C:   AAATTTAAATAATTCATTACAAGCCTTTATTAAA
Fragment F:   CTGCCTCCCAGAGTATTAGGATTACAGGCATAAG
```

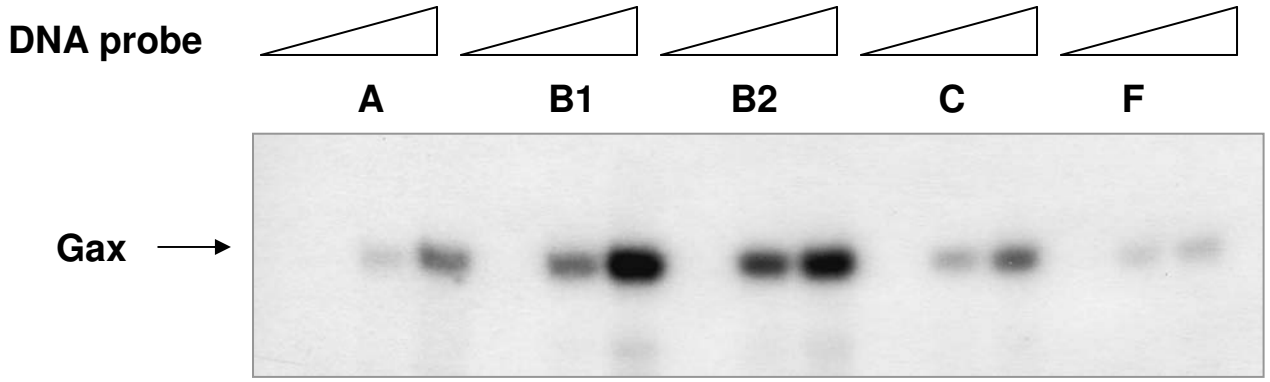
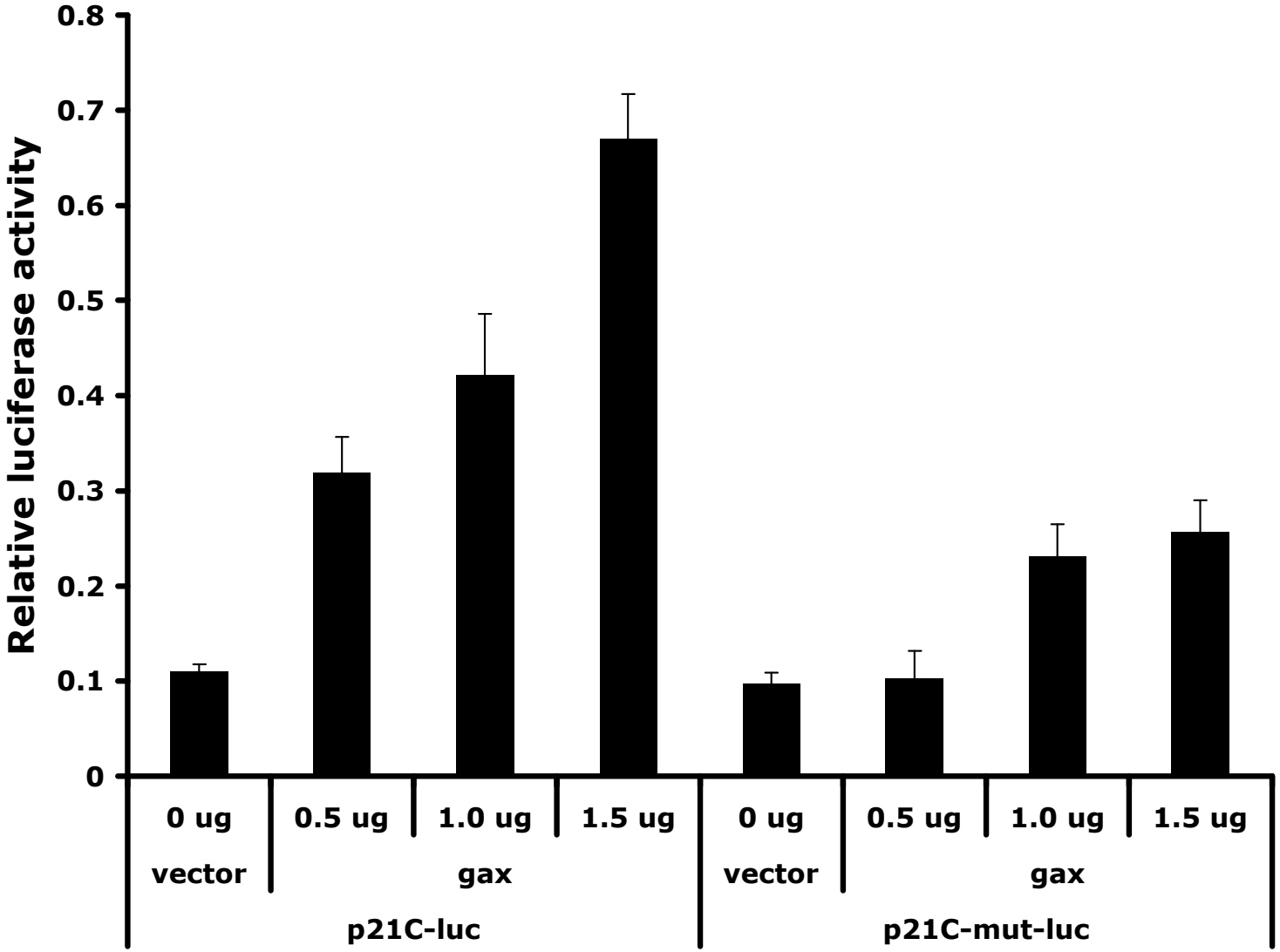


Figure 8B


Relative luciferase activity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 ug
vector
0.5 ug
1.0 ug
1.5 ug
gax
p21C-luc
0 ug
vector
0.5 ug
1.0 ug
1.5 ug
gax
p21C-mut-luc